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AN ECOLOGICAL STUDY OF HEXACHLOROBUTADIENE (HCBD)

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AN ECOLOGICAL STUDY OF HEXACHLOROBUTADIENE (HCBD)

ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF TOXIC SUBSTANCES
WASHINGTON, D.C. 20460

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16. ABSTRACT

Hexachlorobutadiene (HCBD) has been found in the environment in southeastern Louisiana in addition to other parts of the world. In this region it is a byproduct of the petrochemical industry. HCBD has been used as an insecticide in central European orchards and vineyards where excessive contact has caused illness in humans. In this study, soil, water and organism samples were collected periodically in 1974 and 1975 from sites in southeastern Louisiana, with emphasis along the Mississippi River and an industrial region of known contamination of HCBD near Geismar, Louisiana. Maximum HCBD concentrations in water from the two areas were 1.9 and 4.7 µg/ℓ (ppb). Maximum HCBD concentrations in soil from the two areas were 790 and 1,080 µg/Kg (ppb). Laboratory experiments with the compound included acute toxicity studies in aquatic systems and through injection in fish and crayfish. Accumulation and depuration rates were determined and observations made with histological slides of tissue. Other potential measures of stress were made, including blood cortisol levels and oxygen uptake rate. Accumulation in fish was erratic and fairly low, with concentrations normally well below 50X. In algae and sediment, concentration factors were between 200X and 300X. In water, LC_{50}'s were found above approximately 2mg/ℓ (ppm) for fish and crayfish. Exposure to HCBD in ambient water caused histological damage to hepatopancreases of crayfish and liver and kidney of bass. It also elevated serum cortisol level in bass.

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I. SUMMARY AND CONCLUSIONS

The purpose of the study summarized in this report was to determine the distribution and toxic effect of hexachlorobutadiene (HCBD) in selected aquatic systems and organisms. Field collections of soil and water were made in southeastern Louisiana, and were supplemented by samples of aquatic organisms from sites where available. In laboratory experiments, the principal animals used were the red swamp crayfish, Procambarus clarki, the sailfin molly, Poecilia latipinna, and the largemouth black bass, Micropterus salmoides. Both acute and chronic effects of HCBD were studied.

LC_{50}'s were established for organisms in flow-through experiments which utilized a modified proportional diluter. Twenty-five to 30-hour LC_{50}'s resulted when sailfin mollies were challenged with 4.2 to 4.5 ppm (µg/ℓ)HCBD. Concentrations of 1.2 to 1.7 ppm HCBD resulted in LC_{50}'s of 77 to 138 hours for bass. HCBD concentrations of 2.7 and 2.9 ppm resulted in 57-hour and 80-hour LC_{50}'s, respectively, for adult crayfish. When dissolved in oil, HCBD produced lethal effects following administration by injection. Twenty-four hour LD_{50}'s resulted from injections of 3.4 mg/g body weight in crayfish and 1.7 mg/g body weight in Gulf killifish.

The compound did not accumulate to high levels in test animals, and rates of uptake as well as distribution in organs were irregular. Light microscope observation of histological slides of tissues from chronically exposed fish and crayfish revealed damage at the cellular and organ level. Changes were observed in liver and kidney of bass exposed to 32.8 ppb HCBD for 10 days. Hepatopancreas in crayfish was affected by 10 days' exposure to 3.7 ppb HCBD.

Analysis of blood samples taken from freshly-sacrificed fish revealed no differences in hematocrit following chronic exposure to HCBD; however, serum cortisol levels were observed as indicators of stress. HCBD significantly elevated cortisol level in bass exposed to 3.43 ppb HCBD for ten days.

Oxygen consumption rates were not significantly altered by HCBD within three hours after exposure. Chronic exposures produced complex response patterns which were not resolved satisfactorily in the present experiments with fish and crayfish. A flow-through system was used to determine uptake of the compounds by algae (Oedogonium cardiacum) and sediment. Concentration factors remained below 300X for both during the relatively short-term experiments. A food-chain study compared the relative accumulation effects on a predator feeding upon HCBD-contaminated food fish and a similar predator taking up the compound both through its food and through contaminated water. Uptake of HCBD was erratic and bass accumulated more from water than from food when taken as an average.

Laboratory experiments were compared with patterns of accumulation under field conditions. HCBD-free crayfish were caged, placed at a contaminated field site and removed periodically for GC analysis, or for laboratory depuration and subsequent GC analysis. HCBD was not concentrated to a great extent, and accumulated irregularly. Concentration factors for crayfish left in the field site for 17 days ranged from 7.8 to 300X (33.7 to 1,290.3 µg/Kg HCBD). The great majority of HCBD accumulated in specimens during a 10-day exposure was lost following 12 days' depuration.

Since HCBD has been found as a contaminant in the environment, it is possible that it is degraded or altered by ambient UV light. The environment was simulated in the laboratory and the resultant photoproducts formed following short exposure to UV light were analyzed by GC. Irradiation of HCBD yielded several products of higher molecular weight which decrease in number and concentration with exposure time.

II. INTRODUCTION

The present study was initiated following the observation, in recent years, of excessive levels of HCB in adipose tissue and milk of cattle being raised in the vicinity of an industrialized region bordering the Mississippi River between Baton Rouge and New Orleans, Louisiana. HCBD was found to be a co-contaminant of the processes causing these problems. The field portion of this study was undertaken to measure HCBD levels in the environment in southeastern Louisiana. Laboratory experiments were aimed at observing acute and chronic effects of the compound under controlled conditions.

Hexachlorobutadiene (HCBD), a chlorinated compound, is a volatile dense liquid often found in association with HCB as an industrial by-product. Many studies of its toxicity are oriented toward its use as a controlling agent for insect pests, e.g., the vine louse (Khokhryakova et al., 1963), the housefly larva (Levinson, 1955), and the apple tree borer (Vashchinskaya, 1971). Subsequent changes in the microflora following application have been noted (Khokhryakova et al., 1963, Mirzonova and Perov, 1968, Mukasheva, 1974).

Toxic effects of HCBD have been observed in metabolic processes of rats (Gudumak, 1968) following ingestion. Jacobs et al. (1974) noted accumulation of the compound in fatty tissue of rats, and Dmitrienko (1972) observed kidney damage in rats fed HCBD. Murzakaev (1963a) provided LD_{50} values for HCBD in mice, rats and guinea pigs and reported effects upon the central nervous system of rats subjected to prolonged exposures of low doses (Murzakaev, 1967). In the aquatic environment, effects of HCBD upon fish behavior were reported by Hiatt et al.(1953), and Stroganov (1968) presented toxicity data for fish (Leucaspius delineatus) exposed to HCBD.

Russian farm workers were found to suffer several maladies as a result of exposure to HCBD fumigation (Krasnyuk et al., 1969). These, and other consequences of agricultural uses of HCBD have prompted studies to determine maximum permissible limits in water supplies (Murzakaev, 1963b, 1964) and air near industrial sites in the USSR (Poteryaeva, 1972).

In the present study, field work was divided into two distinct phases. First, wide-ranging collections of water, soil and organisms were made to establish levels and distribution of HCBD in the southern central portion of the state of Louisiana, extending eastward to Mississippi and following the Mississippi River from Baton Rouge to Port Sulphur. Results from this work provided an overview of environmental reality against which gross exposures could be compared. The study of the extreme in environmental exposure was concentrated in the immediate vicinity of the Vulcan Materials Company at Geismar, La. Here a periodic monitoring of HCBD concentrations in soil, water and selected aquatic organisms at various trophic levels was conducted. During the course of field work, localities in this latter area were contaminated at a fairly constant level and afforded the opportunity for observation in an environment having otherwise relatively natural conditions.

In an effort to define acute and chronic effects of HCBD upon local fauna more precisely, laboratory experiments were designed to expose aquatic biota to the test compound. Histological preparations, behavioral observations and gas chromatographic analyses were used to obtain data and gain a greater understanding of the significance of the presence of HCBD in the environment.

Tests of acute toxicity have been central to studies of contaminants introduced into environmental systems. Tarzwell (1966, 1971) has discussed the use of acute toxicity levels and of application factors in setting safe standards for levels of toxic substances in natural waters. It has been obvious for a long time that the concept of application factors is more of a convenient means of dealing with the complicated problems of water quality than an accurate scientifically established criterion. Long term effects of toxic substances are difficult to determine in actual practice. The importance of the problem of water pollution, however, is sufficiently great to justify attempts to approximate a relationship between short term lethal effects relatively simple to measure and long term effects which are far more difficult to determine.

Because of the relative ease with which the experiments can be conducted, there have been numerous experiments designed to determine the level of a lethal factor that can be tolerated by a given percentage of animals for a given period of time. Warren (1971) has discussed this subject and also has reviewed the use of application factors in conjunction with tolerance studies. The rationale for incorporating tolerance studies into the present project is that because of the physiological studies included in the project there is a preliminary basis for suggesting application factors for HCBD. The physiological and morphological indications of long term effects of sublethal concentration of HCBD are difficult to establish and such studies are relatively rare. As Warren points out it is extremely important to establish application factors which can be applied with some scientific basis.

Three commonly-used methods of introducing a potential toxicant into an organism are: (1) direct injection, (2) oral feeding, and (3) contaminating its air or water environment. In some experiments during the present study, animals were injected with the test compound and in others they were subjected to a range of concentrations of HCBD in aquatic systems. Condition of animals was observed regularly, and any abnormal behavior or appearance was noted for inclusion as possible pathological effects of HCBD.

Mortalities give the most positive, visible evidence that a substance is toxic to organisms. In contaminated natural systems, however, concentrations of toxic substances are normally below lethal levels. The variable conditions in nature often preclude positive determination of chronic effects of a given substance upon resident flora and fauna. Controlled laboratory conditions are helpful, therefore, in assessing various responses of an organism to low-level exposures. Results of such work can provide a basis for field observations and augment acute tests in establishing application factors.

Chronic tests and observations reported here dealt with a variety of physiological and morphological parameters. Uncontaminated organisms were brought into the laboratory and adapted to in vitro conditions for periods of from two days to as much as two months before being used in tests. Most

animals were subjected to HCBD in the flow-through system, the diluter, which is discussed in Section IV.C. Following exposure for specified periods, whole organisms, or excised organs served as material for GC analysis of uptake and differential distribution in chronic studies. Light microscopic examination of histological slides prepared from excised organs provided further information on chronic effects of HCBD. Samples of blood taken from fish were used in determinations of hematocrit and cortisol levels. Cortisol levels were determined by competitive protein binding radioassay (Murphy, 1967, 1971) which has been successfully employed in teleost plasma (Hargreaves and Porthé-Nibelle, 1974). Alterations in these two levels would be considered an indication of stress upon the animals. Significant changes in these parameters might provide a further means of monitoring conditions in a given area of potential contamination.

Rate of oxygen utilization by an organism may vary in response to stress. In order to study possible effects of HCBD in ambient water of fish and crayfish, respirometry experiments were carried out in the laboratory.

Accumulation and clearance of HCBD in whole-body samples of fish and crayfish and samples of algae and mud are best accomplished in a flow-through system. The modified proportional diluter was used for these exposures and depurations. Concentrations of HCBD in diluter tanks were comparable to levels found in contaminated natural systems, except for some experiments, in which higher concentrations were used. Samples of fish or crayfish being tested were removed from all tanks according to schedules outlined in discussions of the various experiments in the text which follows. Handling of specimens and preparation of extracts for GC analysis are described in the Gas Chromatography unit (IV.D) in the Laboratory Methodology section of this report.

A series of field experiments with crayfish provided comparative information to results from laboratory studies. Uptake and clearance of HCBD was determined in animals, initially free of the compound, which were placed in an HCBD-contaminated field locality.

Experiments were designed to observe possible breakdown products of HCBD. The substance was subjected to UV light and the resultant photoproducts determined.

III. FIELD STUDIES, METHODOLOGY

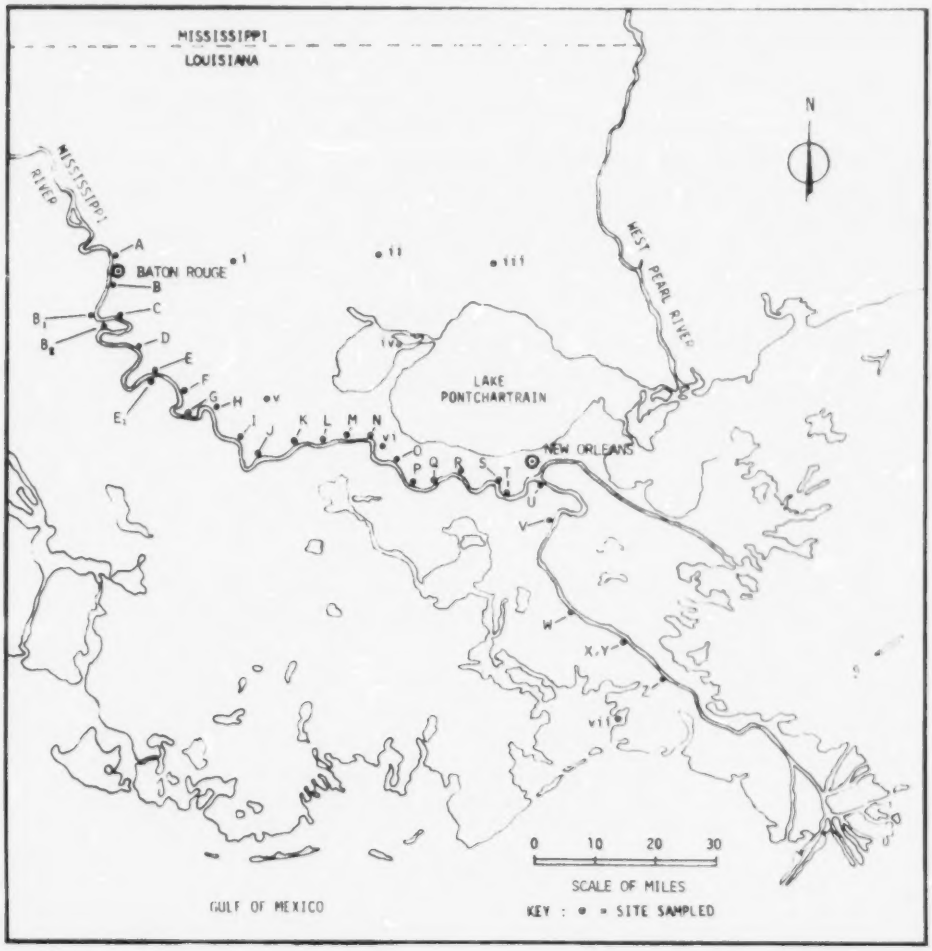
A. Overview

To develop a transect of contamination along the Mississippi River, collections of samples were made at five-mile intervals between Baton Rouge and New Orleans and at greater intervals from New Orleans south to Port Sulphur (Fig. 1). These samples were collected between March and May, 1975. At each site approximately 1 liter of water was taken from about 15 cm beneath the surface of the Mississippi River near the river's edge. A specimen of levee soil was also collected from the river's edge. These samples were taken from beneath the water surface whenever possible. Mud samples were taken from the bottom of ditches running parallel to the levee on the inland side. Fish and aquatic invertebrates were collected from these ditches whenever they were

present. All animal specimens were wrapped in aluminum foil and frozen immediately on dry ice for later analysis. The localities for these field sites are shown in Table 1.

In addition to the samples taken along the Mississippi River, collections were made at several inland sites. These samples were used to provide baseline information on geographic distribution of the compounds. These localities are also shown in Table 1, designated by numbers in italics.

Figure 1. Localities sampled for presence of HCBD in soil and water.



Legend to Figure 1.

Sites

A - T	East bank of Mississippi River
$B_1B_2E_1$, U-Z	West bank of Mississippi River
i, ii, iii, v	Inland, east of Mississippi River
iv	Pass Manchac, inland tidal lake
vi	Spillway of Mississippi River, connected to Lake Pontchartrain
vii	Bay

Table 1. Field localities for overview collections.

	Location Description	Type of Sample: Water*	Soil*	Biota**	Type of Organism
A	Hwy. 90, North of Baton Rouge (E)	X	X	X	F
B	Intersect, River Rd., La. Hwy 327 (E)	X	X		
B_1	Addis at La. Hwy. 990 & La. 998 (W)	X	X	X	F
B_2	Plaquemine at La. Hwy. 988 & La. 1148 (w)	X	X	X	C, F
C	5 Mi. South of B (E)	X	X	X	C, S
D	Sunshine (E)	X	X	X	C, F
E	Carville (E)	X	X	X	C, F
E_1	La. 405, 4 mi. South of White Castle (W)	X	X	X	C, F
F	Ashland Plantation (E)	X	X	X	F & C
G	Darrow (E)	X	X	X	C
H	Intersect, La. 44 & La. 942 (E)	X	X	X	C, F
I	Romeville (E)	X	X	X	F & C
J	Convent (E)	X	X	X	C
K	Lutcher (E)	X	X	X	C
L	Garyville (E)	X	X	X	F
M	Reserve (E)	X	X	X	Sh
N	Laplace (E)	X	X	X	C, F, S
O	South of Bonnet Carré Spillway (E)	X	X		
P	Destrehan (E)	X	X	X	C, F
Q	St. Rose (E)	X	X	X	C, Sh
R	River Ridge (E)	X	X	X	C
S	New Orleans, River Rd. at Causeway (E)	X	X	X	F, Sh, S
T	New Orleans, Audubon Park (E)	X	X		
U	Lower Algiers (W)	X	X		
V	Belle Chase (W)	X	X	X	C, F
W	Myrtle Grove (W)	X	X	X	C, F
X	Mile Marker 42 (W)	X	X	X	C
Y	.8 Mi. South of X (W)	X	X		
Z	Port Sulphur (W)	X	X	X	C
i	Walker (I)	X	X	X	C
ii	Hammond (I)	X	X	X	F, Sh, Cl
iii	Covington (I)	X	X	X	C
iv	Pass Manchac (T)	X			
v	Sorrento (I)	X	X	X	C
vi	Spillway (S)	X	X	X	C, F
vii	Lake Grand Ecaille (B)	X	X		

Key

E: East bank of Mississippi River
W: West bank of Mississippi River
I: Inland, east of Mississippi River
T: Tidal lake
S: Spillway
B: Bay

Key

C: Crayfish
Cl: Clam
F: Fish
S: Snail
Sh: Shrimp

* : GC analyses performed on soil and water samples from all locations.
**: GC analyses performed on biota from selected locations (Ref. Tables 3 & 4).

B. Area of higher concentration

The site of known contamination was near the Mississippi River, between New Orleans and Baton Rouge, at Geismar, La. (Fig. 2). The locations of samples taken on the property of the Vulcan Materials Company in Geismar are presented in Figure 3. These samples were collected during September and December, 1974, and May, August and October, 1975. Water and mud samples were taken regularly, and aquatic organisms were collected whenever available. These specimens were preserved by freezing for analysis in the laboratory.

The first site, an impoundment referred to as Recreation Pond, covers approximately one-fourth of an acre and is located in the north sector of the property (Fig. 3). The man-made pond is 20 feet deep with steep edges characterized by scattered patches of rooted and floating vascular plants. The ecosystem here more closely resembled a natural one than the other sites sampled at the contaminated locale. Zooplankton were abundant in the water column. Oedogonium sp., a common green alga provided food and substrate for microcrustacea. Two common aquatic plants support and protect higher organisms. These are Chara and Najas, in whose mats we collected snails, crayfish, dragonfly larvae and small fishes. These animals feed on the plants and their epiphytic and epizoic components. The small fishes, breeding populations of mosquitofish (Gambusia affinis) and sunfish (Lepomis macrochirus), are food for largemouth black bass (Micropterus salmoides) which were stocked as sport fish. At the Recreation Pond, samples of soil, water, aquatic plants, invertebrates and fishes were collected and analyzed to determine HCBD content in abiotic and biotic components of this environment.

The second sampling site was adjacent to the hex waste disposal area where waste containing HCB and HCBD are now buried. This sampling site was a newly dug pond measuring approximately 50 by 100 feet, 20 feet in depth. Ground water keeps this pond approximately 2/3 full of water. Eventually this pond is destined for waste disposal and burial. During the study, little vegetation was found in the water, but populations of mosquitofish were common there and were sampled regularly along with soil and water. Clean crayfish were brought in, caged, and left in this pond in a field study of HCBD uptake.

The third sampling site was located along a small stream carrying runoff water from the field adjacent to the plant to a small stream in the south ditch. Rainfall determined water level in the south effluent ditch, and the only fishes living there were the mosquitofish and a few mollies (Poecilia latipinna). Crayfish (Procambarus sp.) were taken occasionally, along with regular samples of water, mud and fish.

Organisms were collected on several occasions, and their HCBD content determined and tabulated. Concentration factors were computed as a function of HCBD level in water at the time they were taken. When organisms from several dates of collection were combined, their concentration factor was computed by taking a weighted average of their HCBD content and dividing it by the mean concentration in water samples taken on the same collection dates.

Plankton samples resulted from a tow of a 21 cm plankton net across the Recreation Pond, just below the surface. The material was refrigerated at 3°C in pond water after collection. All organisms settled to the bottom of the holding jar, and were pipetted off. They were then blotted on Whatman #1 filter paper, and subsequently treated with the same methodology as described for algae samples in the Methodology Section (IV.D.) of this report. Concentration is expressed in terms of wet weight of solid material.

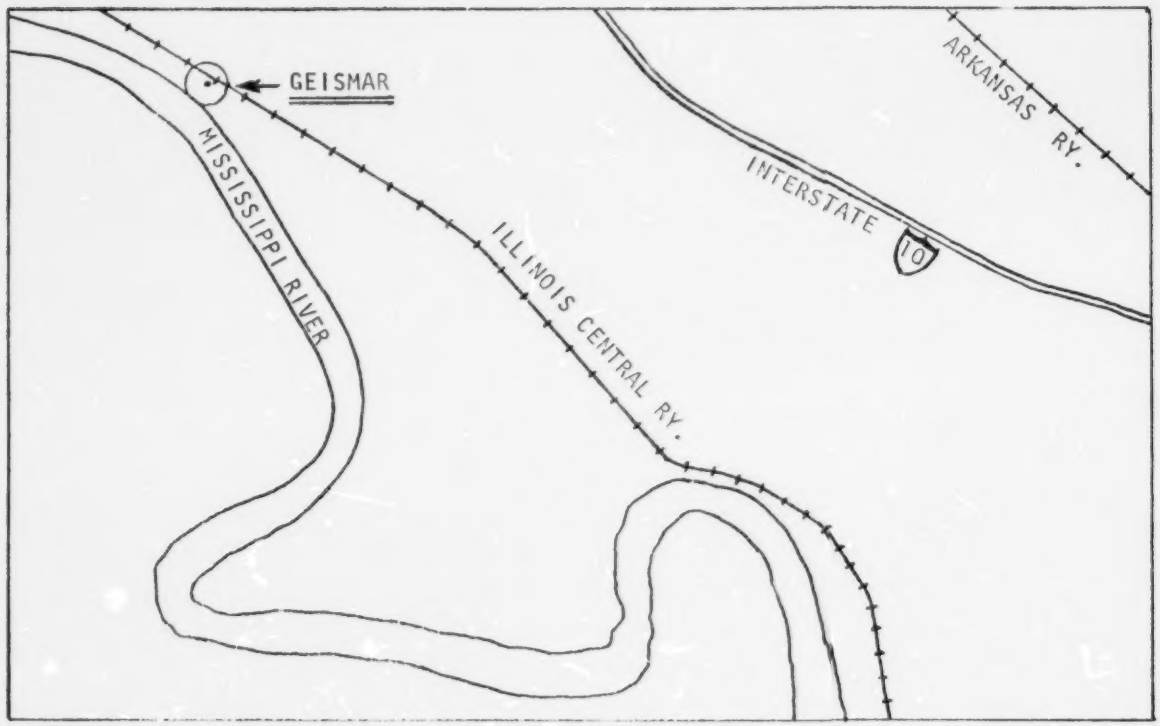
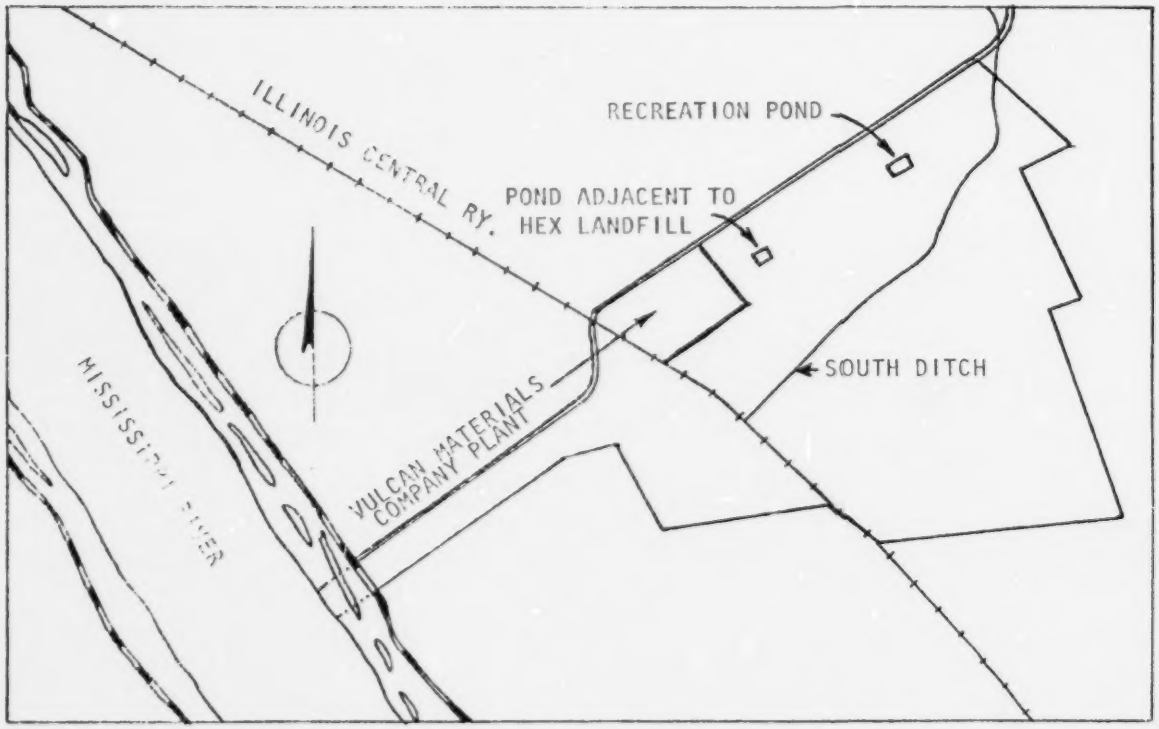


Figure 3. Location of Geismar field sites (area of higher concentration).



IV. LABORATORY METHODS AND MATERIALS

A. Test Compound

In order to guarantee the composition of test compounds following their absorption into organisms it is necessary to analyze their purity before application in various test systems. Zone refined 1,3-hexachlorobutadiene (HCBD) was obtained from Chem Service, West Chester, Pa. 19380. A purity check via gas chromatography was made using a standard of 0.1 ppm of the pesticide in benzene solvent. The compounds found to be "pure" were used throughout all experiments.

Mass spectra were obtained for the compound using a duPont 21-491 double focusing 90° magnetic sector mass spectrometer (MS) equipped with a Bell and Howell Datagraph 5-134 galvanometer driver recording oscillograph. Hexachlorobutadiene was introduced via the batch inlet system. All spectra were obtained at 70 eV at a source temperature of 200°C. Computer plots of the spectra were obtained by use of a PDP-12 LDP computer and a D1100 Versatec electrostatic printer plotter. A mass spectrometry plot of HCBD starting material is given in Figure 4. Figure 5 represents gas chromatographic traces of the two compounds, HCB and HCBD.

Figure 4. Mass spectrometry plot of HCBD.

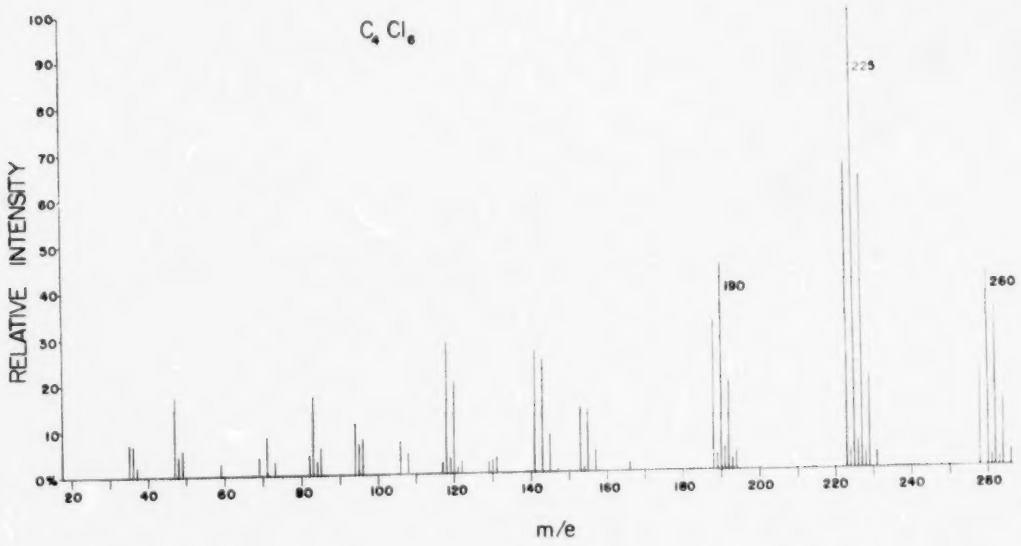
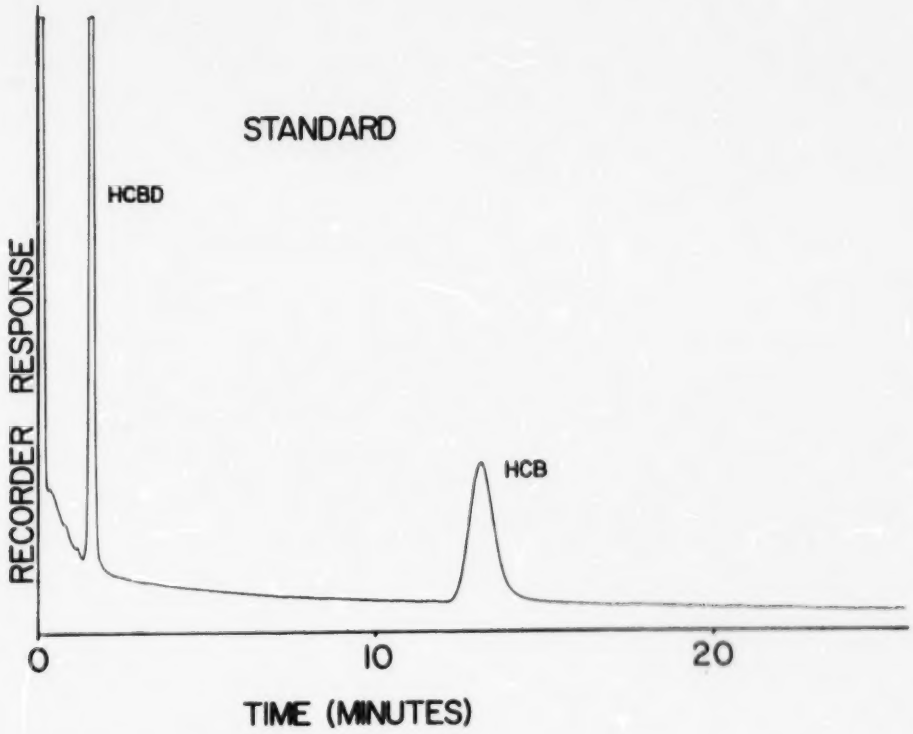


Figure 5. Gas chromatographic traces of HCB and HCBD (chromatographic conditions as described in Section IV.D.).



B. Culture Techniques

Due to seasonality of some forms or habitat preferences of others, experimental organisms used in laboratory studies were acquired from several sources. In a given experiment, however, organisms were all from the same source.

The original stock of filamentous green algae, Oedogonium cardiacum (IU #39), was received from the Indiana University Culture Collection at Bloomington, Indiana. Subsequent cultures were maintained under controlled conditions in aerated flasks containing Bold's Basal Medium in the algae culture room at the contractor's institution.

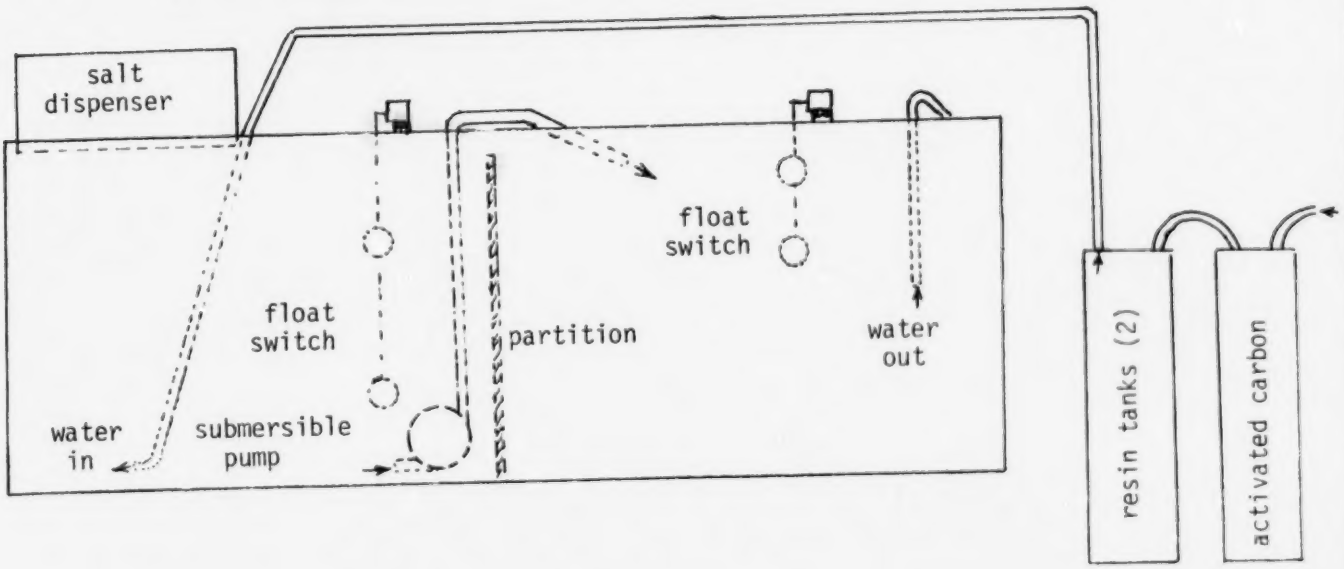
Crayfish (Procambarus clarki) were received as available from commercial facilities at The Crayfish Farm, Sorrento, Louisiana. They were maintained in fiberglass tanks filled with enough water to keep gill regions wet. Crayfish were kept at room temperature and fed HCBD-free chicken meat and small fish.

Small estuarine fishes, including sailfin mollies (Poecilia latipinna) and sheepshead minnows (Cyprinodon variegatus) were collected by seines and dipnets as needed for experiments and food. These were taken in small canals and ditches at Irish Bayou, inland from the southeastern shore of Lake Pontchartrain, Orleans Parish, Louisiana. Grass shrimp (Palaemonetes sp.) and small crayfish (Procambarus sp.) were also found at this locality. These organisms were maintained in filtered, aerated aquaria at room temperature. Salt levels were held at 4 to 5 ppt with Rila Marine Mix (Rila Products, Teaneck, N.J.) artificial sea salts.

Largemouth bass (Micropterus salmoides) were provided by the Louisiana Wildlife and Fisheries Commission through their hatchery at Alexandria, Louisiana. The fish were held in 300 - liter tanks in filtered, aerated, room-temperature water at a level of .8 to 1 ppt salinity. Initially, bass were fed a commercial fish food (Purina Trout Chow) until it was found to contain HCB residues at levels up to 80 ppb. Small fish from Irish Bayou collections served as bass food during subsequent work.

Experimental animals and the water from which they were taken were analyzed for HCBD content prior to acceptance for routine use. All fish and crayfish were maintained in the same quality water, with salinity adjustments as needed. Water for stock holding tanks and experiments was prepared in the following manner. Chemical residues in the New Orleans tap water, including chlorine, were reduced by passing water through an activated carbon bed and deionizer tanks. Deionized water was aged approximately 24 hours in holding tanks, diagrammed in Figure 6. Ionic balance was restored and standardized with the addition of Rila Marine Mix to a level of 1 ppt. An automatic salt mix dosing apparatus assured constant salinity level. This device also administered sodium bicarbonate which maintained the pH between 6.65 and 7.9.

Figure 6. Water Treatment system.



C. Static and Flow-Through Assay Systems

Aqueous experiments were of two basic types, static and flow-through. Static tests with mollies (Poecilia latipinna) were carried out in five-gallon glass jugs filled approximately half full with 10ℓ of prepared water at 1 ppt salinity. The test compound, dissolved in nanograde acetone was pipetted into the jugs concurrently with pouring water to effect mixing. Test fish were added and air above the water's surface was saturated with flowing oxygen before the covers were screwed shut. In this type of experiment, water was left unchanged but oxygen was added periodically during the test. Later static tests involved replacement of water and toxicant no more frequently than once daily.

Static tests with crayfish (Procambarus clarki) involved placing the animals in individual finger bowls. Water and toxicant in acetone carrier were replaced daily. The animals were held in environmental chambers with light and temperature controls for the duration of experiments.

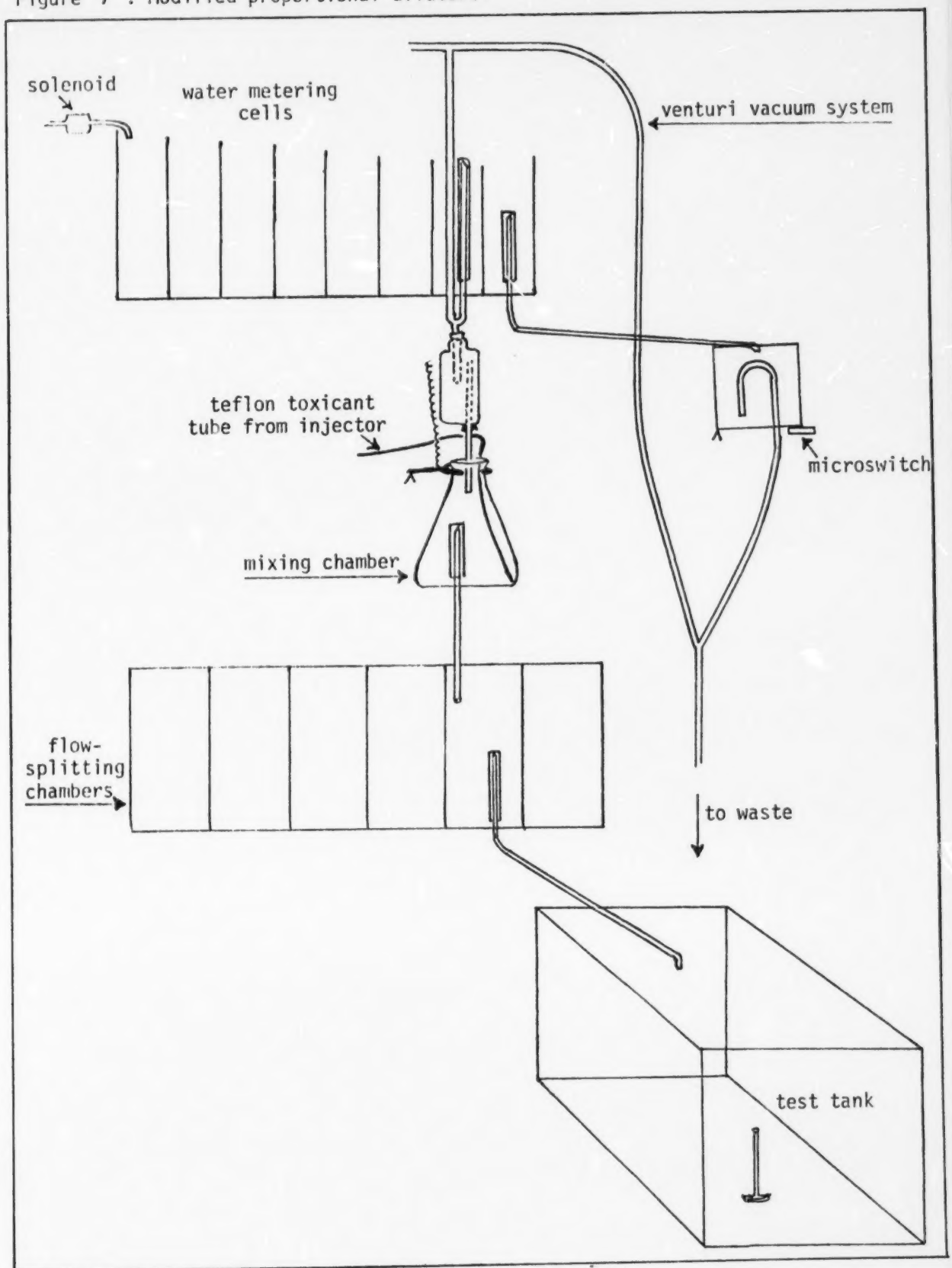
The flow-through aqueous system more closely simulated the natural environment because water with a predetermined and constant load of test compound and acetone carrier was flowing at regular intervals into tanks containing the organisms. This system received water from the tank source discussed in Methodology section IV.B. and functioned as a modified proportional diluter. The design for this apparatus was based upon developments by DeFoe (1975) and preceding workers (Benoit and Puglisi, 1973, Mount and Brungs, 1967). In the modified proportional diluter (Fig. 7), water from a single source fills a series of seven glass chambers in stepwise fashion. Once full, a self-priming siphon in the final chamber initiates a flow of water in the venturi vacuum system. The partial vacuum thus created empties a pre-set, constant volume of water from each of the filled chambers through a siphon whose action is started by the partial vacuum.

The flowing water from each chamber fills a flask (mixing chamber) mounted upon a lever extending from an injector apparatus developed by George J. Frazer *. The flasks are suspended by spring tension. Weight of the water filling the flask depresses the lever, actuating an advance rachet whose pressure hub bears upon the plunger of a 50 ml syringe. A known volume of test compound and acetone carrier is injected into the flask (mixing chamber) through an elongate teflon needle. Once filled, the mixing chambers each empty automatically into flow-splitting chambers through self-priming siphons. Other siphons within each flow-splitting chamber deliver the water to respective test tanks through glass tubing.

Each of the six major test tanks (Fig.8) have a filled capacity of 70ℓ, but were usually filled to a level of 30ℓ as controlled by an adjustable

*: 4528 Pitt St., Duluth, Minn. 55804.

Figure 7 . Modified proportional diluter.*



* one of six similar units schematically represented.

Figure 8. Major test tank.

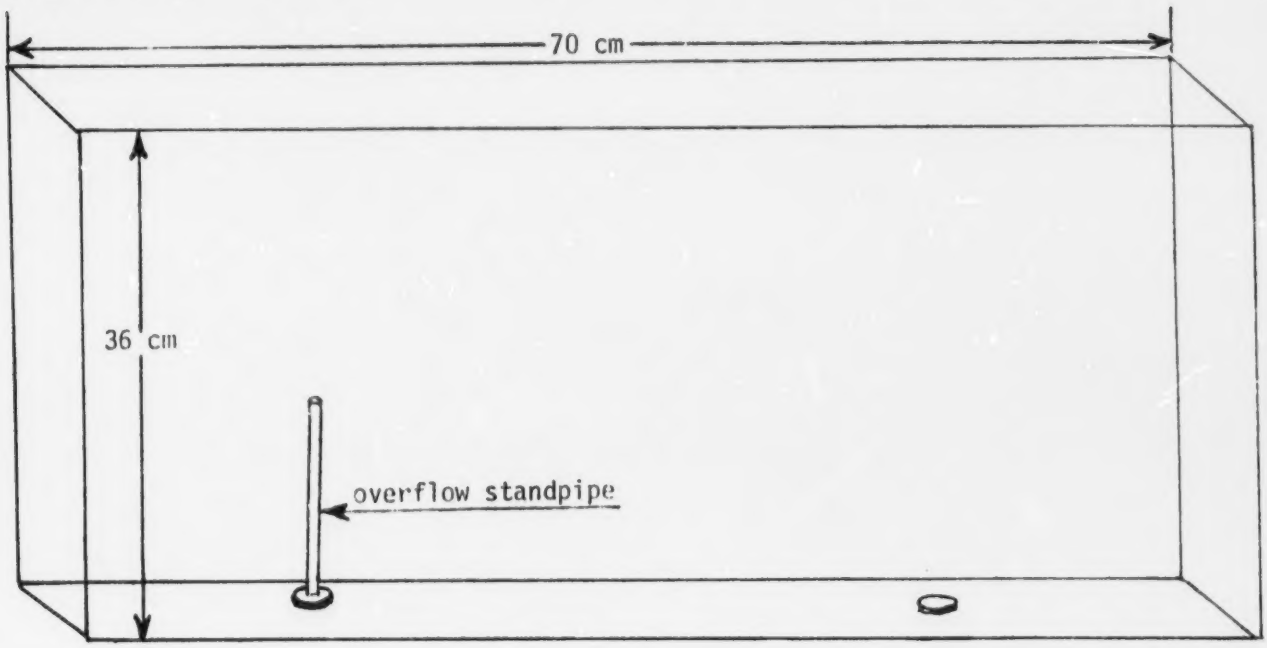
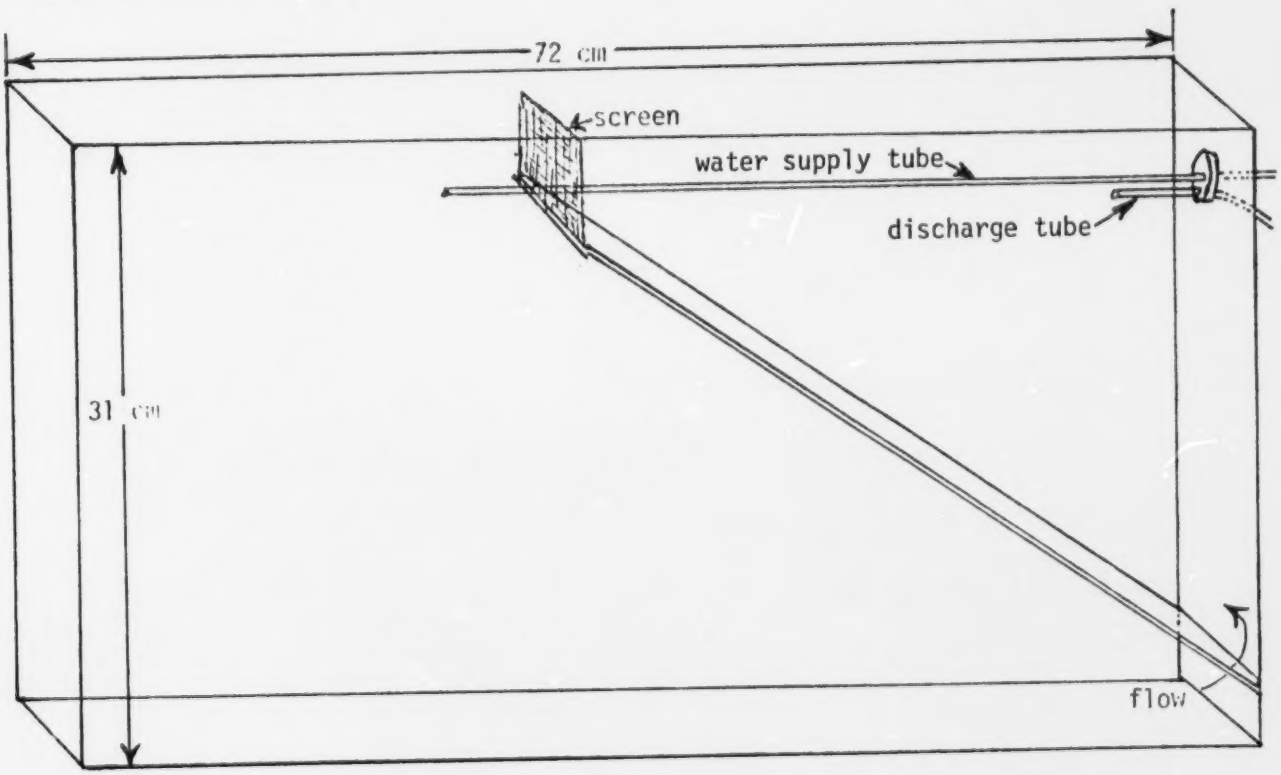


Figure 9. Model ecosystem.



overflow standpipe. The smaller tanks (Fig. 9) are model ecosystems in which water flows into the smaller space, containing mollies, through a gap at the base of the partition. Eventually water exits at the discharge tube which maintains a volume of 30ℓ in the tanks. Flow rate was fixed such that each experimental tank received 60 to 120ℓ of water daily.

The protocol for most tests provided for two duplicate test tanks of each of two compound concentrations in addition to a water control and an acetone carrier control. The number of organisms per tank was usually determined by the number and type of samples needed for analysis. Availability of animals and their tolerance to conditions were other factors contributing to this figure.

HCBD levels in tanks were checked by analysis of water samples siphoned from beneath the surface. In certain experiments, organisms were removed according to a fixed schedule during both phases, uptake and depuration. Each phase usually lasted 10 or 15 days for each test. In other experiments, all animals were sacrificed at the same time, 10 days after initiation of exposure. Variations in routine will be discussed in appropriate sections of this report. Temperature, pH, and dissolved oxygen content of tank water were monitored regularly during flow-through experiments. These ranged as follows: temperature, 22.2 to 23.9°C; pH, 6.5 to 7.9; and oxygen, 7.6 to 8.5 ppm.

The greater portion of acute experiments utilized the flow-through system with HCBD in the test animals' aquatic medium. Some injection experiments, however, were carried out with HCBD dissolved in peanut oil. A dosage of .01 cc/g body weight was injected directly into the hemocoel of each animal, at the base of its second walking leg on the left side. Several concentrations of HCBD were used. In an effort to achieve a more uniform dispersion of HCBD to be injected into the hemocoel of crayfish, we prepared an emulsion as the carrier of HCBD. HCBD was first dissolved in peanut oil. The oil was then sonicated with lecithin as an emulsifier in 0.38 M sucrose solution.

Fish were also used in injection experiments. In the absence of sufficient numbers of bass, the Gulf killifish (Fundulus grandis) was selected for use in acute injection experiments. A stock solution of 1.2 ml HCBD was dissolved in 6 ml of peanut oil. Dosages administered intraperitoneally were 3.4, 1.7, .8 and .2 mg/g body weight, and an oil control, injected at the rate of .01 ml/g body wt. Each injected group consisted of 10 fish. Following injection, fish were held in HCBD-free water in diluter tanks until they succumbed. Some survivors, including the controls, were kept for more than six weeks.

Flow-through conditions of the diluter were useful for two other types of experiments; specifically, those in which algae and sediment were tested.

To test uptake of HCBD by a common filamentous green alga, a culture of Oedogonium cardiacum (I.U. #39) was prepared. Original stock was grown in 4ℓ flasks in 3ℓ of 1:1 3N BBM: H_2O solution. Aeration was not provided. Illumination in the culture room was provided by Sylvania lifeline fluorescent bulbs (output 3150 lumens) operating on a 12:12 light: dark photoperiod. Approximately 40 days after inoculation of the stock flask, the algae was divided into four relatively equal portions, three of which were placed into open glass jars and submerged in three tanks (model ecosystems) of the diluter. The fourth was used in an HCB experiment.

Protocol for diluter tanks consisted of acetone and water controls and 16.9 ppb HCBD solution. Illumination to the tanks was provided by three Sylvania lifeline

fluorescent bulbs (40 watt; output 3150 lumens) placed at right angles to the tanks' long axes, one lamp located centrally 20 cm above water surface, and two lamps 10 cm behind tanks, +8 cm and -16 cm from water surface. Trace elements present in 1 ppt Rila marine mix-reconstituted deionized water were complemented by metabolic products of four mollies in a separate portion of each tank.

Samples of the algae were collected following one day of exposure and subsequently on every other day through a period of two weeks. Samples were removed from each tank by pipet and placed in a cone of filter paper to drain off excess water. Damp clumps of algae were stored in glass vials and refrigerated at approximately 3°C. Preparation of samples for GC analysis is discussed in section III.D. of this report.

An experiment was designed to measure uptake of HCBD by soil in the form of sediment in the bottom of a test container. For both test and control flasks 200 g samples of dry soil collected at a farm in Talisheek, Louisiana, and determined by GC to be free of HCBD, were screened through 1.6 mm mesh aluminum screening and poured into the bottom of 1000 ml aspirator flasks. At the flow rate of 3 l/hr, water originating in the diluter passed through a glass tube into the bottom of the stoppered flasks. Circulated water left through a waste tube attached to the aspirator arm at the neck of the flask. Samples of 40 g (wet wt) of mud were removed from the flasks periodically and stored in glass jars at approximately 3°C until they were extracted for analysis on GC.

Laboratory experiments with crayfish were supplemented with a series of field experiments. Animals initially free of HCBD were placed in cages and set on the bottom of a pond adjacent to the hex waste disposal area at the highly elevated field site discussed in Section III.B. HCB and HCBD content of the water was monitored. Periodically, crayfish were removed from the cages and either frozen for later analysis of uptake or returned to the laboratory and maintained in clean water in environmental chambers. Samples of crayfish were removed at intervals from the environmental chambers, and extracts made from them provided depuration data for animals exposed to both compounds in an environment further complicated by the natural products of the industrial wastes.

D. Gas Chromatography

Preparation methods for samples prior to gas chromatographic (GC) analysis depended upon the substance being extracted. In preparation of water samples, aliquots of 350 ml were shaken with 20 ml of benzene for 3 hr on an Eberbach reciprocating action shaker. Following passage through a separatory funnel an aliquot from the benzene layer was ready for injection into the GC. In preparing mud, aliquots of approximately 20 g of mud were shaken with 20 ml of acetone for 20 minutes, after which 20 ml of benzene was added. Following 24 hr of shaking, the benzene-acetone extract was injected into the GC.

Samples of algae were scraped from walls of the tanks and blotted on filter paper. Each sample was weighed, sonicated in acetone in an ice bath to disrupt cell walls, and subjected to the same procedure used for animal tissue. Concentrations of HCBD were expressed in terms of wet weight of the algae.

Samples of animal tissue were weighed and homogenized with anhydrous sodium sulfate and acetone. The liquid was filtered into a separatory funnel and the residue homogenized twice with acetone which was then added with filtration to the separatory funnel. After adding sodium chloride to the combined acetone extracts in a separatory funnel, the acetone-sodium chloride mixture was extracted three times with hexane and the hexane evaporated to near dryness on a rotary evaporator. This residue was dissolved in hexane and placed on a Florisil column washed previously with 50 ml of elution solvent (95% hexane, 5% ether). Following elution with 100 ml of elution solvent the eluent was evaporated on a rotary evaporator. The residue was dissolved in 10 ml of benzene and an aliquot was prepared for injection into the GC.

All extracts of water, soil, algae and tissue samples were analyzed by a Hewlett Packard 5710A gas chromatograph (GC) equipped with an electron capture detector utilizing ^{63}Ni foil. Extracts were introduced by a 7671A automatic sampler. This system was attached to a Hewlett Packard 3352B Laboratory Data System. Separation was accomplished with a 91.44 cm X 4 mm I.D. 10% OV-1 column maintained at either 150° or 165°C depending upon the integration method used. Argon-methane 95:5 was employed as the carrier gas at a flow rate of 35 ml/min. The injection port temperature was held at 250°C and the detector was set at 300°C.

Quantification was accomplished using external standards of 1,3-hexachlorobutadiene at concentrations of 1 ppm and 0.1 ppm in a benzene solvent. Concentrations of HCBD in water were computed in terms of µg/ℓ of sample, and expressed in parts per billion (ppb). Other samples reported in µg/g are expressed in parts per million (ppm).

E. Mass Spectrometry

A double focusing duPont 21-491 mass spectrometer was employed which was attached to a Hewlett-Packard 5750 gas chromatograph and coupled to a PDP-12-LDP computer. Separations were carried out isothermally on a 9 m x 4 mm I.D. glass column packed with 10% OV-1 stationary phase on acid washed chromosorb. Transfer lines were maintained at 200°C. All spectra were obtained at 70 eV at a source temperature of 200°C.

F. Corticosteroid Analysis

The fish were maintained on a 12:12 (light:dark) photoperiod (lights on at 0730) for the entire duration of exposure to HCBD. On the final day, blood was collected between 2 and 3 hours after "dawn." Blood was taken from the sinus venosus into heparinized hematocrit tubes which were then sealed with clay and centrifuged. The tubes were cut and the plasma separated and stored in a freezer until used.

Plasma corticosteroid concentration was determined using a modification of the competitive protein-binding radioassay technique described by Murphy (1971) which has been used successfully in teleost plasma (Hargreaves and Porthé-Nibelle, 1974, Meier and Srivastava, 1975). A 10 µl aliquot of plasma was expelled into a centrifuge tube containing 1.0 ml absolute ethanol. The ethanol precipitates plasma proteins which might interfere with the assay (including any endogenous

corticosteroid binding globulin) and also extracts the cortisol. The tubes were centrifuged and a 0.5 ml aliquot of the supernatant ethanol containing the cortisol was transfered to a reaction vessel and evaporated to dryness under N_2. Each sample was then incubated for 5 minutes in a water bath at 45°C with 1.0 ml of corticosteroid binding solution. Each 100 ml of this solution contained 0.5 μCi of 1,2H^3-cortisol (obtained from New England Nuclear Corp.) and 5 ml of pooled male horse serum (obtained from the LSU Veterinary School) with distilled water to volume. Horse serum was used as a source of corticosteroid binding globulin (CBG) since Ficher et al. (1973) have found that it gives greater specificity for cortisol.

After incubation, the samples were transfered to an ice bath for 30 min and then 40 ml of Florisil was added to absorb the unbound cortisol. A 0.5 ml aliquot of each sample was counted and compared to a standard curve obtained by measuring known amounts of cortisol.

G. Fish Blood Hematocrit

The animals used in these studies were exposed to various concentrations of toxicant. They were bled on the tenth day of exposure, first being stilled by immersion in ice cold distilled water. Blood was taken from the sinus venosus into heparinized hematocrit tubes which were then sealed with clay and centrifuged. Packed cell volumes were read using a Critocap® micro-hematocrit tube reader.

H. Respirometry

The effect of exposure of juvenile crayfish and mollies to HCBD was measured using a Gilson Respirometer (Unbreit, et al., 1972). Each set of tests using juvenile crayfish (average weight = 10 mg) employed animals from one hatching of one female. Juvenile mollies (average weight = .3 g) were collected from uncontaminated environments and used immediately after collection.

Five crayfish were placed in each respirometer vessel in 3 mls of water. In the experiments using mollies a single fish was placed in each vessel in 7 mls of water.

Animals were placed in the respirometer, and following 15 minutes of acclimation, oxygen consumption was monitored. In crayfish studies, respiration rates were determined every 15 minutes for two consecutive 1 hour periods. The system was flushed with air after the first hour.

The rates of respiration of mollies were determined every 10 minutes over two thirty-minute periods. The system was flushed with air following the first-thirty-minute period.

Animals were placed in covered 8-inch finger bowls in either control or experimental conditions during exposure periods. The culture solution was changed daily. Mollies were kept in deionized water reconstituted to 2.5 ppt with artificial sea salt. Crayfish were kept in deionized water reconstituted to 1 ppt. The experimental groups were exposed to water that had been stirred with appropriate amounts of HCBD for at least 24 hours before the animals were placed in the solutions.

The solution in each respiration vessel was the same as that to which the individual animal or group of animals had been exposed. Therefore, respiration rates were determined during exposure. Respiration rates were determined for HCBD immediately following exposure and after several longer intervals of exposure. The concentrations are given in the "Results" section (V.E.). Oxygen consumption rates were expressed in terms of wet weight of the animals.

I. Histology

Fingerling largemouth bass and crayfish for histological examination were selected at random from the inhabitants of each tank of the modified proportional diluter system to represent water control, acetone control, and each nominal level of HCBD utilized for GC analysis. Animals were sacrificed on the day of termination of toxicant accumulation. Brain, green gland, hepatopancreas, one gill and a sample of abdominal muscle were excised from each crayfish.

Largemouth bass were stilled by chilling, weighed, and total and standard lengths measured. The liver was excised and weighed as another measure of size and nutritional condition and fixed. The right kidney, the first right gill arch, approximately 5 mm of epaxial muscle caudal to the right operculum, and a segment of intestine immediately caudal to the stomach were excised and fixed. Organs were examined under dissecting microscope magnifications and any grossly damaged areas noted. In one experiment, each animal utilized for histological study was also subjected to GC analysis.

Tissue samples were fixed in neutral buffered formalin, Bouin's, or Zenker-acetic, appropriately washed, dehydrated, cleared in toluene, and infiltrated and embedded in 56°C - 58°C paraffin. Tissue blocks were serially sectioned and mounted. Slides representing step sections were stained in Harris' hematoxylin and eosin or Lillie's modification of Weigert's iron hematoxylin and eosin. All mounted sections were kept to permit study of serial sections of any areas found on microscopic examination to be of specific interest.

J. Photochemistry

Solutions of HCBD in both hexane and benzene (1 µg/10 ml) were irradiated at varying time intervals. HCBD was irradiated for 15 minutes, 30 minutes and 60 minutes. Irradiations at 253.7 nm were conducted in serum capped quartz test tubes employing a Rayonet RPR-100 Chamber Reactor equipped with 16 8-watt lamps and a "merry-go-round" apparatus (The Southern New England Ultraviolet Co., Middletown, Conn.) All samples were degassed by purging with nitrogen for a period of 10-15 minutes prior to irradiation.

V. Results

A. Field Studies

Field work consisted on two distinct phases. The first, a regional overview, dealt with HCBD residue determinations in water, mud and aquatic organism samples collected along a Mississippi River transect from Baton Rouge to Port Sulphur, and other parts of southeastern Louisiana. The second phase, at an area of higher concentration, was located on property of the Vulcan Materials Company in Geismar, Louisiana. Specific localities and protocol are discussed in the Field Methodology section of this report.

1. Overview

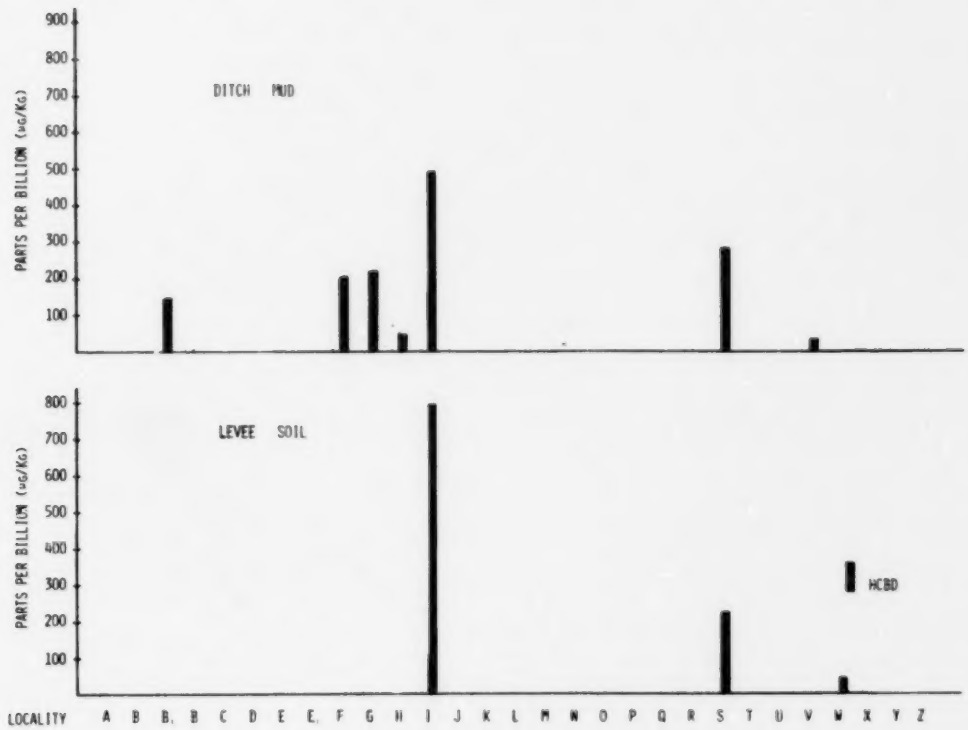
Figure 1 shows localities in the immediate vicinity of the Mississippi River sampled for HCBD residues in collections of water, mud, crayfish and fish made during the first phase of field work. Concentrations of HCBD in mud and soil are presented in Figure 10. Supplementary sites are outlined in Table 1 in the Field Methodology section. Comparisons of HCBD residues in soil, water and organisms from various sites can be seen in Tables 2 through 4.

2. Area of Higher Concentration

The area near the Vulcan Materials Company plant in Geismar, Louisiana, was the site of a series of field collections during 1974 and 1975. Three specific localities were selected for sampling of water, mud and organisms. These sites, designated Recreation Pond, South Effluent, and Landfill Pond (pond adjacent to hex landfill) are discussed in detail in section III of this report. Table 5 gives concentrations of HCBD residues in water, mud, and organism samples taken during several seasons of the year.

Chara and Najas are two plants found in the pond. The former, an alga, is less plentiful, has a high calcium carbonate content and attaches to the bottom. The latter occurs as floating, unattached masses in all parts of the pond. Eleocharis, an emergent plant, was taken in shallow water less than .3 meter deep, near the shoreline. The snail Physa was common in masses of Najas. Anisoptera were represented by dragonfly larvae taken while dipnetting in floating vegetation near the shore. Procambarus sp., the crayfish, was taken periodically during scoops of the dipnet that took up some bottom sediment as well. Gambusia, the mosquitofish, was more frequently found near the surface in shallow water, while juvenile sunfish, Lepomis, less than 4 cm in length, lived in the protective cover of aquatic vegetation. Micropterus, the single bass collected, is a common sport fish in this region.

Figure 10. Distribution of HCBD in soil along the Mississippi River, Louisiana. Baton Rouge - Port Sulphur transect.



CODE	LOCALITY
A	Hwy. 90, north of Baton Rouge
B	Intersect. River Rd., La. Hwy. 327
B_1	Addis at La. Hwy. 990 & La. 998
B_2	Plaquemine at La. Hwy. 988 & La. 1148
C	5 mi. south of B
D	Sunshine
E	Carville
E_1	La. 405, 4 mi. south of White Castle
F	Ashland Plantation
G	Darrow

CODE	LOCALITY
H	Intersect. La. 44 & La. 942
I	Romeville
J	Convent
K	Lutcher
L	Garyville
M	Reserve
N	Laplace
O	South of Bonnet Carré Spillway
P	Destrehan
Q	St. Rose

CODE	LOCALITY
R	River Ridge
S	New Orleans, River Rd. at Causeway
T	New Orleans, Audubon Park
U	Lower Algiers
V	Belle Chasse
W	Myrtle Grove
X	Mile marker 42
Y	.8 mi. south of X
Z	Port Sulphur

Table 2 . Concentrations of HCBD in water and soil samples from sites removed from Mississippi River transect.

Location	Code	Water HCBD in µg/l (ppb)	Soil HCBD in µg/Kg (ppb)**
Walker	(i)	.7	*
Hammond	(ii)	1.2	*
Covington	(iii)	*	*
Pass Manchac	(iv)	1.5	*
Sorrento	(v)	.7	*
Spillway	(vi)	1.5	321.5 (433.0)
Lake Grand Ecaille	(vii)	1.0	21.6 (62.6)

*: < .7 ppb
**: Figures in parentheses are corrected for dry weight of sample.

Table 3 . Mean concentrations of HCBD in Mississippi River mosquitofish (Gambusia affinis) in comparison with levels measured in water and soil.

Location	Code	Water HCBD in µg/l (ppb)	Soil HCBD in µg/Kg (ppb)**	Fish HCBD in µg/Kg (ppb)
Garyville	(L)	.9	*	112.8
Romeville	(I)	1.4	793.4 (1001.3)	197.4
Baton Rouge	(A)	1.9	*	827.3

*: < .7 ppb
**: Figures in parentheses are corrected for dry weight of sample.

Table 4 . Mean concentrations of HCBD in crayfish (Procambarus sp.) from ditches in comparison with levels measured in soil.

Location	Code	Soil HCBD in µg/Kg (ppb)**	Crayfish HCBD in µg/Kg (ppb)
Walker	(i)	*	10.6
Romeville	(I)	489.9 (665.3)	24.1
Ashland	(F)	204.9 (410.1)	70.1
Darrow	(G)	219.4 (420.2)	22.9

*: < .7 ppb
**: Figures in parentheses are corrected for dry weight of sample.

Table 5. HCBD residues in water, mud and organism samples from area of higher concentration.
(Number of organism samples in brackets; Figures in parentheses corrected for dry weight of mud samples).

Organism	# of analyses	Mean HCBD concentration in µg/Kg(ppb)	Concentration factor	Abiotic Component	Dec. 1974	April 1975	May 1975	Aug. 1975	Oct. 1975
Recreation Pond									
Plankton	1	1016.	2,701x	Water (ppb)	*	1.2	.7	.1	.38
Chara	1	12.	120x	Mud (ppb)	*	190. (270.)		580. (670.)	550. (730.)
Najas	1	9.	13x						
Eleocharis	1	20.	29x						
Physa [3]	1	345.	3,446x						
Gambusia [27]	3	890.	2,225x						
Lepomis [45]	15	600.	1,546x						
Micropterus [1]	1	liver: 2405.	1,093x						
		muscle: 72.	33x						
South Effluent									
Anisoptera [1]	1	315.	7,507x	Water (ppb)	**	**	1.0	.04	.65†
Procambarus [9]	3	997.	1,404x	Mud (ppb)	**	250. (430.)	**	700. (1010.)	1080.† (2370.)
Gambusia [24]	8	817.	1,151x						
Landfill Pond									
Gambusia [27]	9	16,205.	3,525x	Water (ppb)	**	4.6	4.7	*	4.49
				Mud (ppb)	*	380. (500.)	**	*	650. (920.)

* : no sample taken.
**: no value determinable from GC analysis.
† : site changed due to construction.

B. Acute Toxicity

1. Crayfish Injections

Several experiments were carried out in which HCBD, dissolved in peanut oil alone, or prepared as an emulsion, was injected into crayfish. Although acute toxicity was observed in the emulsion injection experiments, GC analysis of the emulsions later indicated large variations in the concentration of HCBD in the injected material. These results from GC indicated an unstable emulsion and rendered the results of six injection experiments useless.

Because of the above difficulties a more direct method was adopted. HCBD was dissolved in peanut oil at three different concentrations. Each animal was injected with .01 cc/g body weight. As in earlier experiments, oil was injected directly into the hemocoel of each animal, at the base of its second walking leg on the left side. Results of this work are given in Table 6 . HCBD was obviously toxic to crayfish at doses in excess of 336 µg/g (ppm) body weight, when injected in an oil medium.

Table 6. Injections of HCBD in oil into crayfish (Procambarus clarki).

Concentration of solution	N injected	µg/g body wt.	LD_{50}	Number of crayfish surviving 6 days
336 ppt	10	3,360	<1 days	0
33.6 "	10	336	5 days	0
3.4 "	10	34	——	7
Oil control	10	——	——	10

2. Fundulus Injections

Dosages of HCBD administered to Gulf killifish through intraperitoneal injection were 3.4, 1.7, .8 and .2 mg/g body weight, and an oil control, all injected at the rate of .01 ml/g body weight. The first three of these concentrations were four-hour, 20-hour and 18-day LD_{50}'s, respectively. No fish died during a three-week period at the lowest dosage, 200 ppm.

3. Crayfish LC_{50}'s

In static experiments, four groups of eight animals each were distributed as follows: water controls, water with acetone controls and experimentals at 5 and 10 ppm, respectively (nominal concentrations of HCBD). Two water control crayfish molted and died during the test. Two acetone control animals died; one of these had also molted first. Two crayfish in 10 ppm jars died; one of these had also molted first. These results do not suggest that HCBD caused death under these particular experimental conditions.

In the diluter, 64 full-size adult crayfish were tested in two tanks (nominal: 10 ppb; GC $\bar{X}$: 2.97 ppb HCBD), 32 animals were in water with acetone and 32 others were water controls. Uptake lasted 10 days and depuration another 10 days. Of a total of 64 crayfish, two experimental animals in a single HCBD tank and two controls in the water control tank died during the test. Their deaths were apparently the result of cannibalism following molting. No deaths, therefore, could be attributed to exposure to this concentration of HCBD.

In another diluter experiment, nine crayfish were held in each of two experimental tanks with GC-measured HCBD levels of 2.7 ppm and 2.9 ppm. Half of the animals were dead within 57 hours in the first tank, while half of the crayfish in the second tank had died within 80 hours. Controls in uncontaminated water and water with acetone carrier survived the experiment.

A static experiment yielded acute toxicity data for juvenile crayfish in HCBD. In a series of animals, each of which weighed approximately 12 to 14 mg, the 70-hour LC_{50} was 5 ppm (nominal concentration).

4. Fish LC_{50}'s

Tests with both bass and mollies were carried out to provide LC_{50} data. The first test with bass was a static experiment in which tanks were kept in an environmental chamber. Temperature was maintained at 25°C and a 12:12 light:dark photoperiod was maintained. Tanks were filled to 10 liters with deionized water reconstituted to 1 ppt salinity with Rila marine mix. HCBD was inoculated directly into the experimental tanks. Droplets remained dispersed in the tank but not dissolved, indicating that actual concentration in water was lower than calculated doses. In this preliminary experiment, three bass each were placed in tanks with nominal concentrations of 40, 20 and 5 ppm µg/ℓ) and a water control.

In these static experiments, a nominal calculated concentration of 40 ppm HCBD killed all three test animals in less than 45 hours. The average body HCBD concentration of two fish analyzed was 2.06 µg/g (ppm). A concentration of 20 ppm killed all three within 70 hours. The HCBD content of one fish analyzed was 2.83 µg/g. All three fish in the tank with 5 ppm HCBD died in less than 96 hours.

Findings from the static experiment were applied to tests in the diluter. The first flow-through experiment used six bass per tank. In one tank, all six animals died within 20 hours of being subjected to 2.5 ppm HCBD, as determined by GC. In another tank, half of the six animals tested were dead within 43 hours and all had died within 48 hours at a measured level of 4.3 ppm HCBD. At the GC-measured level of 2.2 ppm HCBD, all six fish died in less than 114 hours. At a measured level of .9 ppm HCBD, all specimens were distressed within 24 hours, immobilized and inverted in 114 hours, but revived with depuration at the termination of the experiment.

Two bass experiments were carried out at substantially lower concentrations of HCBD. In the first of these, tanks of twelve fish each were subjected to the following conditions: 3.43 ppb HCBD, 31.95 ppb HCBD (both measured on GC; mean of 3 separate analyses), water control and acetone control. All specimens survived 10 days of exposure in the diluter, at which time they were sacrificed and frozen for subsequent GC analysis.

In the second experiment, bass were tested in the following format:

Tank	A	B	C	D	E	F
Number of Fish	11	11	10	10	11	11
Nominal Concentration in ppb (µg/ℓ)	10	10	H_2O Control	Acetone Control	100	100
GC Concentration in ppb($\bar{X}$ of 3)	5.65	7.80	—	—	59.10	57.12

Fish were exposed to toxicant for 10 days and were depurated for 10 days. Groups of bass were removed and sacrificed on days 1, 5, 10, 11, 15 and 20. No fish died due to toxicant levels during the entire test.

The seasonality of young bass made it necessary to continue LC_{50} tests with a substitute species of fish. Two diluter experiments with mollies were directed toward levels known to have lethal effects on bass. Test conditions were the same as for bass, with two notable exceptions. Salinity was increased to 2.5 ppt and tank volume reduced by 50%. The latter change was possible due to decreased biomass of specimens and served as one means to increase turnover rate of aquarium water.

In the first series of experiments a 77-hour LC_{50} of 1.9 ppm (mg/ℓ) HCBD was found for a series of 11 mollies. A 138-hour LC_{50} of 1.2 ppm HCBD was found for a second series of 11 fish. Sixteen specimens had a 77-hour LC_{50} in a tank containing 1.4 ppm HCBD, and another 14 had a 115-hour LC_{50} at 1.7 ppm.

A diluter experiment at higher concentrations of HCBD resulted in LC_{50}'s of less than 48 hours. Twenty mollies were placed in each experimental tank and subjected to a GC-measured level of 4.5 ppm and 4.2 ppm respectively. The LC_{50} in the first tank was attained after 30.5 hours of exposure. In the second tank, half of the animals had died within 26.5 hours.

5. General Pathological Effects

HCBD had acutely lethal effects upon organisms tested during our work. A number of behavioral changes were noted in exposed organisms. Crayfish held in 2.7 and 2.9 ppm HCBD in the diluter appeared sluggish within 16 hours. Some were lying on their sides in the tanks. Following 24 hours, one specimen was lying on its back and died within two more hours. In 1.2 to 1.4 ppm HCBD, mollies were affected in less than 24 hours. Darker pigmentation appeared almost immediately. Within 19 hours loss of equilibrium was observed with some animals lying on their sides and fibrillating the pectoral fins. Others swam in circles and ultimately assumed inverted positions, usually with the trunk curved stiffly to one side. These fish lived another two days in this condition. Some of those affected after 24 hours lived for 10 more days. In 2.5 ppm HCBD, bass were swimming erratically within 18 hours and all had died two hours later. These assumed an inverted position and were gasping during several hours before death. In a lower concentration, .9 ppm, bass were distressed within 18 hours, swimming in circles. They all were inverted within two days and remained alive, gasping, for three more days. The toxicant was removed at this point and fish recovered within 4 days in HCBD-free water. These animals were fed and behaved normally during the week that followed.

In summary, fish showed a consistent pattern of behavior in response to HCBD poisoning. Pigmentation increased, even at sublethal doses. Loss of equilibrium and muscle tetany resulted in a rigid flexing of the body. Fins were held widely spread, and pectorals vibrated rapidly.

C. Chronic Toxicity

1. Crayfish Tissue Morphology - Normal and Pathological

The hepatopancreas of Procambarus clarki and closely related species has been the subject of histological study since the late nineteenth century. (Huxley, 1880) Morphological and physiological studies indicate that the hepatopancreas is analogous to vertebrate liver, pancreas and gut. It functions in enzyme secretion, digestion, and absorption of food and in glycogen,lipid and mineral storage (Fingerman et al. 1967). These facts coupled with reports of liver damage in other species following HCBD exposure led to priority being given to histological analysis of this organ.

The hepatopancreas develops as a bilateral evagination of the midgut. Each unilateral component consists of a short common duct which gives rise to longitudinally oriented central canals of the anterior and posterior lobes. The common duct and central canals are lined with tall, simple columnar epithelium with a striated border. From the central canal numerous diverticula of the eipthelium are derived. These are surrounded by a "basket" of myoepithelial cells and are bound one to another by fine connective tissue.

The lining of each diverticulum is a simple columnar epithelium which shows a variety of cell types from distal to proximal extent. Four or five distinct cell types have been described; but a discussion of the merits of various classifications is not pertinent to the present report (for references see Loizzi, 1971). There is general agreement that the germinative center for

the epithelium lies in the distal-most region of each diverticulum where the epithelium consists of relatively small, slender columnar cells with basophilic cytoplasm (E-cells). These cells apparently give rise to all other cell types, including absorptive, secretory and fibrillar cells which vary cytologically and stain differentially with routine hematoxylin-eosin preparations following Zenker-acetic fixation. Also seen are inclusions which have been described as metals (iron and copper) in the deeply basophilic fibrillar cells (Fe-cells) and in eosinophilic cells (Cu-cells) (Ogura, 1959, Miyawaki et al., 1961). All cell types are found intermingled within the epithelium gradually giving way to large vacuolated columnar cells in the proximal region of each diverticulum. Cells in the proximal region of the diverticulum exfoliate into the lumen and are removed from the gland as a component of the secretion.

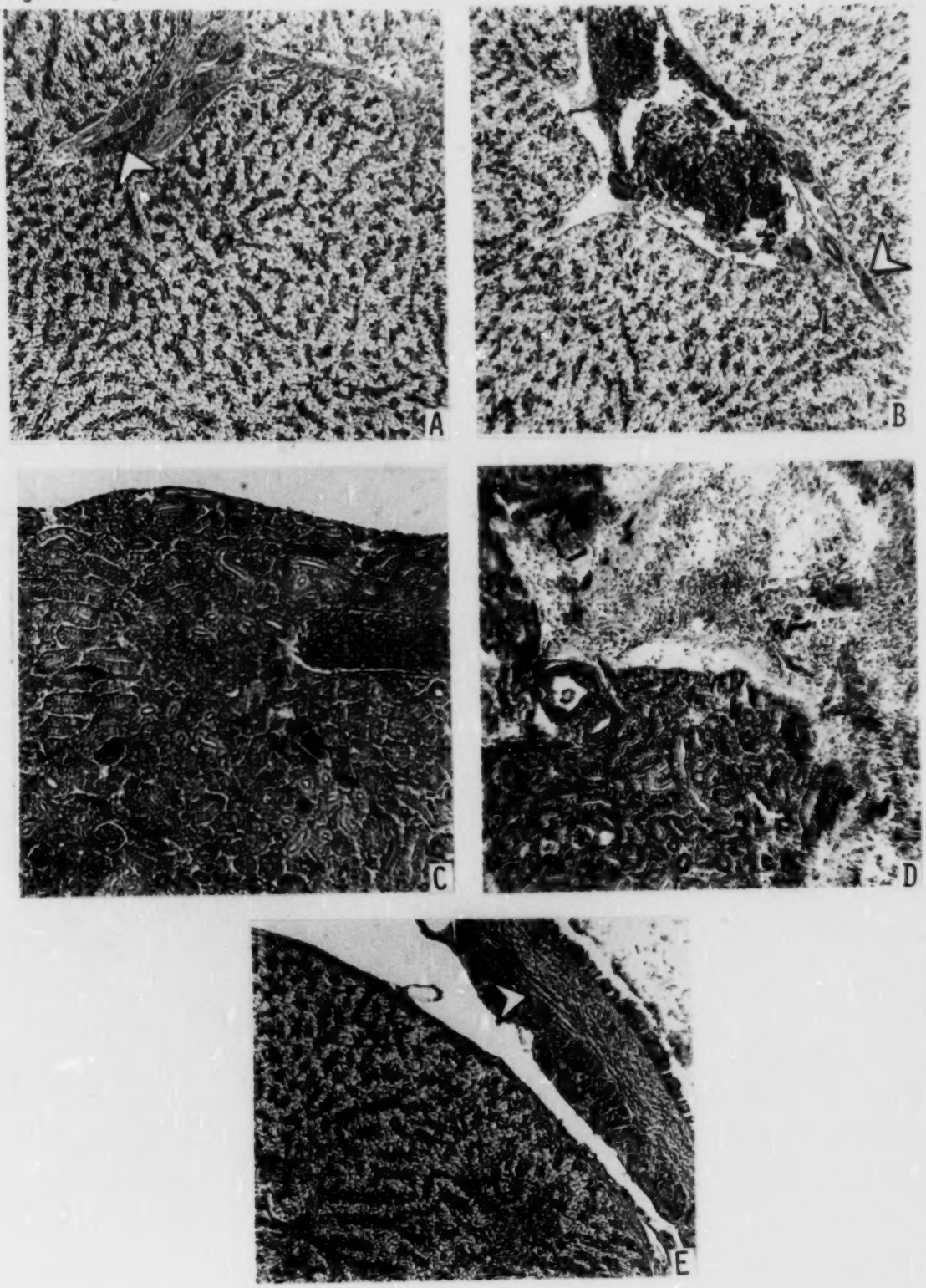
Each green gland (antennal gland) is a nephron-like unit consisting of coelomosac, labyrinth, and nephridial tubule. The tubule leads to a bladder and ultimately to an excretory pore. In Procambarus blandingi light and electron microscope studies (Peterson and Loizzi, 1975) reveal that the epithelial cells of the coelomosac are similar to the podocytes of the vertebrate glomerulus. Epithelium with well-defined brush border comprising the labyrinth indicates a reabsorptive function for this region; whereas the nephridial tubule is lined with epithelial cells lacking a brush border but with basal plasmalemmal invaginations (Beams et al., 1956, Peterson and Loizzi, 1975) such as are found in the distal tubule of the mammalian kidney (Pease, 1955). Thus the organ is considered as excretory and osmoregulatory and in many respects analogous to the vertebrate kidney. For this reason the green glands have been preserved for histological analysis from water and acetone controls, and HCBD-exposed crayfish.

Grossly the majority of hepatopancreases from HCBD-exposed crayfish did not differ from H_2O and acetone controls except in occasional instances of necrosis as noted also for HCB.

Macroscopic examination of the best controlled laboratory environmental groups shows remarkably healthy tissues, for the most part. HCBD distribution in these tissue samples may be considered in conjunction with histological observations. These data are included in the account of an HCBD distribution experiment in section V C3. Tissue appeared healthy upon gross examination, however, there were areas of epithelial exfoliation more distal than normal within the canals and, most importantly, a heightened rate of mitosis in the germinative tips which also contained small areas of seemingly disorganized growth. Such histological findings were seen in animals from seven-day static experiments with a nominal concentration of 5 ppm HCBD in water, and animals from a 10-day diluter experiment with a concentration of 3.7 ppb HCBD in water and a whole-body tissue concentration of 0.173 µg/g HCBD.

These findings would indicate that the crayfish hepatopancreas is a very promising system for further study of longer-term chronic exposure to see the direction that these early indications of abnormal growth may take. Furthermore, the hepatopancreas with its diversified cell population, where function varies with morphological type, may prove to be an excellent cell system for answering more specific questions as to the nature of toxicant damage.

Figure 11, A-E. Photomicrographs of bass tissue sections.



Explanation of Figures

(All figures are photomicrographs at the same mangification, taken of sections stained with H & E.)

A. A microscopic field of fingerling largemouth bass (Micropterus salmoides) liver shows the normal lobulation pattern. Cells interpreted as exocrine pancreas (arrow) are seen in the connective tissue around a large vessel. (H_2O Control Bass)

B. This section illustrates the existence of normal liver lobulation but cytological change with marked eccentricity of pyknotic nuclei and loss of stainability (H & E) of the cytoplasm. The arrow indicates cells interpreted as pancreatic acinar cells in the connective tissue stroma of the portal canal. (Bass exposed to 32.8 ppb HCBD for 10 days in diluter; 10.8 is mean HCBD concentration in tissue. See section VII C4.)

C. A section of fingerling bass kidney illustrates the normal histology. Note the size of the glomeruli and the tubules. (H_2O Control Bass)

D. A section from the kidney of a fingerling bass showing some intact tubules and a portion of a rather large lesion containing extravasated blood. (Bass exposed to 32.8 ppb HCBD for 10 days in diluter; 3.7 µg/g is mean HCBD concentration in tissue. See section VII C4.)

E. Normal liver may be seen on the left and a small segment of the gall bladder wall in section is seen on the right. The arrow indicates the location of pancreatic cells. (H_2O Control Bass)

2. Bass Tissue Morphology - Normal and Pathological

Cursory histological examination of the various excised organs (see Methodology) has indicated that liver, gall bladder, and kidney were of prime importance for histological study in bass.

In the largemouth bass, (Micropterus salmoides), the liver is elongate, conforming to the shape of the pleuro-peritoneal cavity, and a gall bladder is present. The gland parenchyma is seen to be lobulated with the classical lobule easily discerned. Large hepatic portal veins, hepatic arteries, and bile ducts are seen in the interstices of the lobules (Fig. 11A).

The gall bladder lies cupped by a lobe of the liver. It is lined by a simple columnar epithelium with a rather dense fibrous connective tissue layer beneath. The muscularis is distinct. (Fig.11E).

The present study reveals that in this species of fish, acini of exocrine pancreas are situated in the connective tissue around the hepatic portal vein and around the major bile ducts in the hilus of the liver and accompany the hepatic portal vein, hepatic artery, and bile ducts in their distribution within the liver parenchyma (Fig. 11A, arrow). Exocrine pancreas also is situated between the muscularis and serosa of the gall bladder (Fig. 11E, arrow). Exocrine and endocrine cells are intermixed to form small nodules of pancreas in the gastro-hepatic ligament. Variability among fish species has been noted previously in the location of endocrine and exocrine pancreas (Bengelsdorf and Elias, 1950, Bertolini, 1965, Falkmer and Winbladh, 1964, Fujita, 1964, Weis, 1972).

The kidney, an opisthonephros, shows relatively large renal corpuscles and short proximal and distal tubules. A thin loop of Henle is not present (Fig. 11C) Other bass tissues (gill, brain, and muscle) have been excised, examined grossly and scanned histologically. Approximately two hundred blocked specimens are available for further detailed study.

Histopathological change has been noted in one bass (specimen E1 in Tx 37) following HCBD exposure (See Section V C4). It differed from that seen following HCB exposure. Macroscopically, the liver was noted to be pale. On microscopic examination, the parenchyma showed an accentuated lobulation with hepatocytes that were small, with pale eccentric nuclei and an empty-looking cytoplasm (Fig. 11B). The kidneys from TX37-E1 were abnormal showing areas of leucocytic infiltration (Fig. 11D). They were not as damaged as those in HCB tanks. In this same bass, the gall bladder was observed as opaque white, an indication that HCBD may cause damage to this organ similar to the damage found in HCB exposure.

3. Retention and Distribution of HCBD in Crayfish

Distribution of HCBD in crayfish tissue was observed in a experiment which exposed a series of crayfish to 2.97 ppb (μg/ℓ) of HCBD for 10 days. At the end of the exposure period, four animals were sacrificed and their tissue samples grouped and frozen together. Subsequent GC analysis gave the results seen in Table 7.

Table 7. Distribution of HCBD residues in tissues of crayfish (Procambarus clarki) exposed to 2.97 ppb for 10 days.

Tissue	HCBD in µg/g (ppm)	Concentration factor
Brain	35.27	11,875X
Green gland	2.188	737X
Hepatopancreas	.173	58X
Gills	.165	56X
Muscle	.046	15X

4. Retention and Distribution of HCBD in Bass

Distribution of HCBD in various organs of bass was determined in an experiment in which the fish were exposed to two concentrations of HCBD in the diluter in addition to the appropriate controls. Exposure lasted 10 days, at the end of which nine specimens in each tank were dissected and the tissues frozen in groups of three. The groups were extracted and analyzed separately. Resulting data are given in Table 8 and the average values in Figure 12. Highest levels were found in gut, liver, kidney and brain of bass exposed to approximately 33 ppb HCBD. The highest concentration, in the gut, was 52.73 µg/g (ppm), a concentration factor of 1610X.

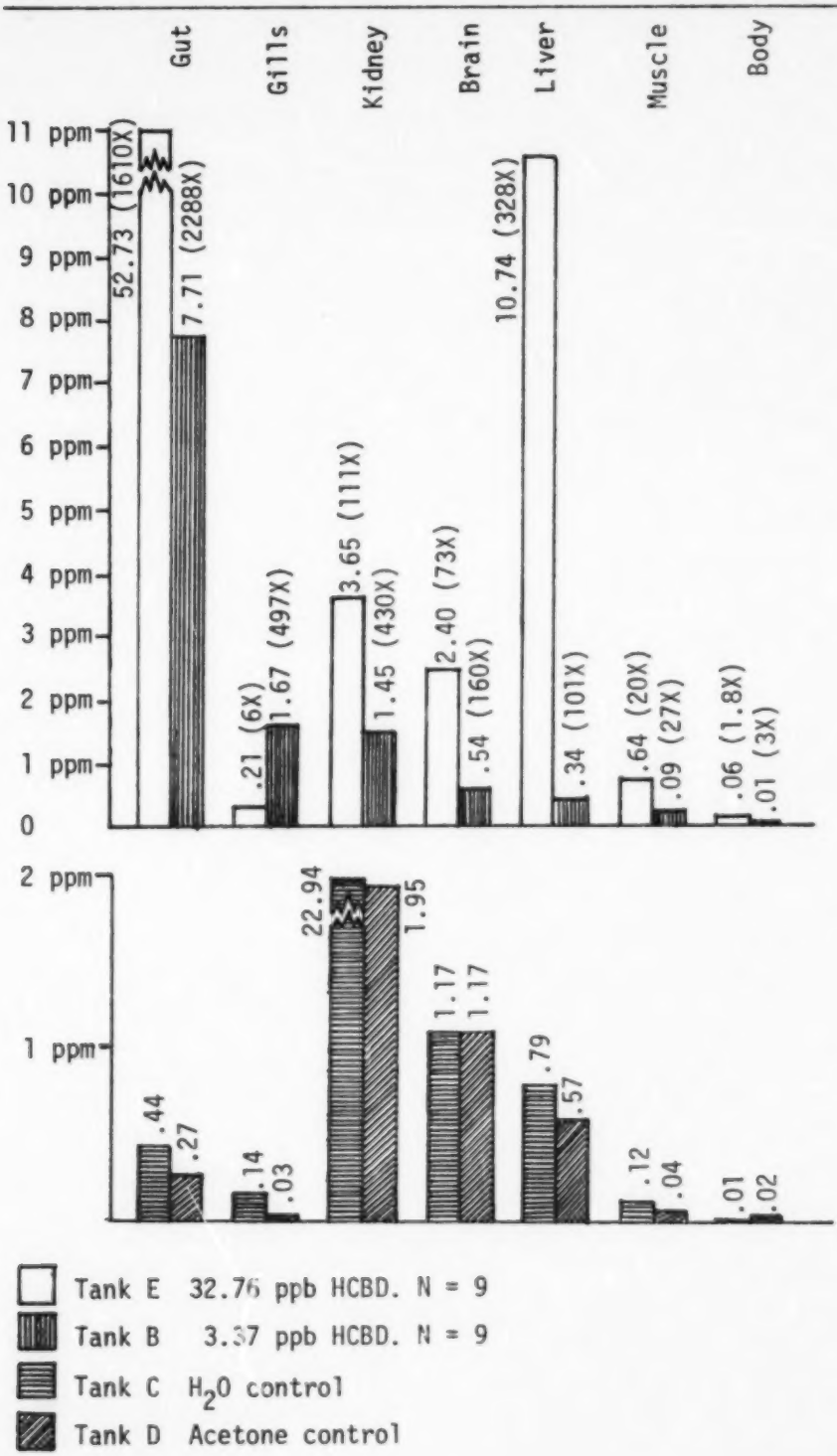
Table 8. HCBD content of tissue samples from bass exposed to HCBD in diluter for 10 days. Values in µg/g (ppm

Tank	Sample Numbers	Organ Type: Gut	Gills	Kidney	Brain	Liver	Muscle	Body
B 3.37 ppb (µg/ℓ)HCBD	4-6	.410	.050	2.575	.970	.417	.172	.008
	7-9	.203	.184	1.574	*	.114	*	.044
	10-12	22.531	4.795	.203	.107	.492	.013	.001
	$\bar{x}$	7.715	1.676	1.451	.539	.341	.093	.018
E 32.76 ppb (µg/ℓ)HCBD	4-6	122.452	.296	6.700	.809	**	.033	.003
	7-9	15.937	.099	1.012	.298	15.208	.070	.142
	10-12	19.803	.250	3.236	6.106	6.282	.089	.023
	$\bar{x}$	52.731	.215	3.650	2.404	10.745	.064	.056
C H_2O Control .74 ppb (µg/ℓ)	4-6	.560	.124	16.810	.851	.371	.035	.004
	7-9	.565	.278	49.384	2.401	1.880	.296	.020
	10-12	.181	.024	2.621	.270	.125	.045	.020
	$\bar{x}$	.435	.142	22.938	1.174	.792	.125	.0147
D Acetone Control 1.0 ppb (µg/ℓ)	4-6	.510	.012	.989	1.510	.010	*	.064
	7-9	.035	.035	1.139	*	1.513	.076	.001
	10-12	.257	.056	3.752	.841	.207	.004	.008
	$\bar{x}$	.267	.034	1.96	.784	.577	.04	.024

* : below detectable levels.

**: sample lost during processing.

Figure 12. Average HCBD concentrations of tissue samples from bass exposed to HCBD for 10 days. Concentration factors in parentheses.



5. Fish Blood Hematocrit

Twelve bass were maintained in test conditions given in Table 9 for a period of 10 days. The individual variation and mean values do not indicate an effect upon hematocrit by HCBD at the concentrations tested.

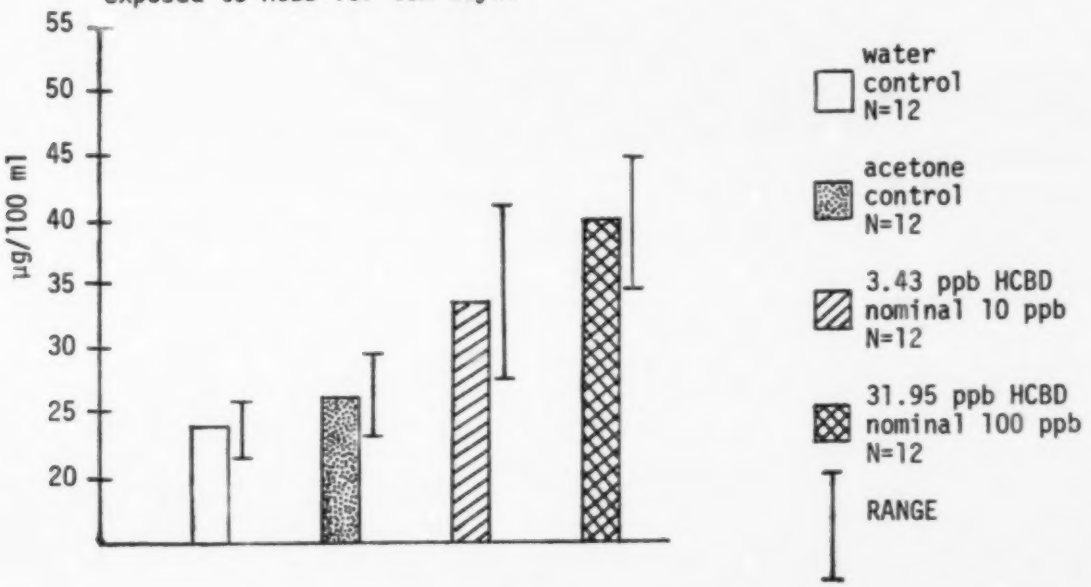
Table 9. Hematocrit of bass (Micropterus salmoides) exposed to HCBD

TANK	C	D	B	E
Conditions	Water Control	Acetone Control	4ppb nominal 3.43ppb (GC)	40ppb nominal 31.95ppb (GC)
$\bar{X}$ Hematocrit (%)	27.85	24.83	28.0	23.23
range (%)	17-33.8	14.3-30.9	20-33.8	12-34

6. Fish Corticosteroid Level

Figure 13 shows the results of an assay of plasma cortisol levels (µg/100 ml) in bass exposed to HCBD for ten days. A t-test shows that the mean values for the control groups are significantly lower (5% level) than those of the experimentals (exposed to 3.43 ppb and 31.95 ppb) indicating that the experimentals were under more stress than the controls. Since experimental conditions were exactly the same for all groups except for the concentration of toxicant these results indicate a significant stressful effect of HCBD.

Figure 13. Mean plasma cortisol levels (µg/100 ml) in bass (Micropterus salmoides) exposed to HCBD for ten days.



D. Accumulation and Clearance

It is an established fact that many contaminants accumulate at different levels in different abiotic substances and in organisms at various trophic levels in an ecosystem. Biomagnification has been observed numerous times. With this in mind, the present study was designed to analyze HCBD levels from several parts of the natural system. Levels of accumulation were observed in field samples, and laboratory tests were carried out for comparative purposes and to establish rates of accumulation. Components of the simulated ecosystem included sediment, algae, crayfish and fish. The algae, Oedogonium, is a genus found at the contaminated study site, and serves as food for such grazers as snails (Physa), crayfish (Procambarus), mollies (Poecilia latipinna), and mosquitofish (Gambusia affinis). These invertebrates and fishes are common food items of larger predators, including sunfish (Lepomis) and bass (Micropterus). Several organisms named above were used in a number of laboratory experiments discussed here, in an effort to increase available knowledge of accumulation of HCBD in the food chain.

1. Crayfish

In the diluter, 64 crayfish were tested in two tanks (nominal: 10ppb; GC $\bar{x}$: 2.97 ppb HCBD). Thirty-two animals were acetone controls and 32 others were water controls. Uptake lasted 10 days and depuration another 10 days.

Two males and two females were removed from each tank following 1,5 and 10 days of exposure and 1,5 and 10 days of depuration. Whole bodies of males and females were processed and analyzed separately for each removal date. Data from this experiment are presented in Table 10 and Figure 14.

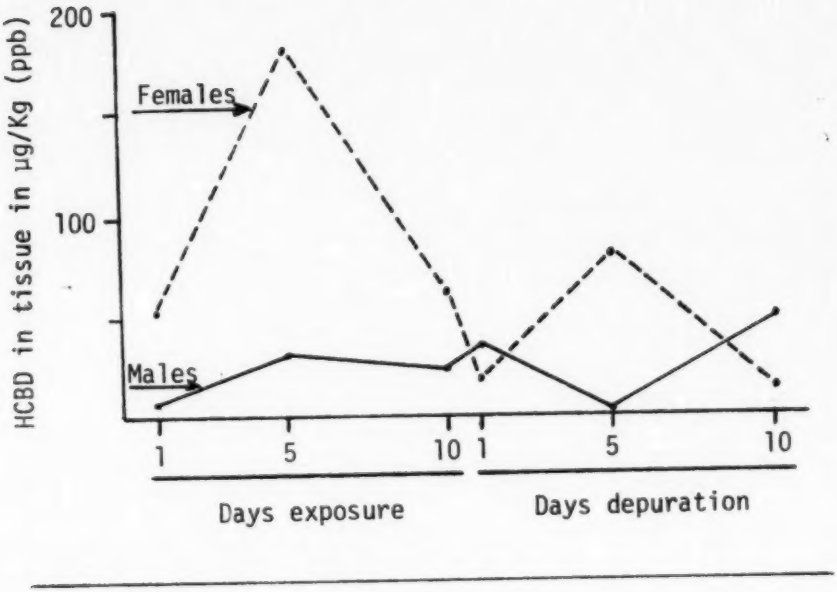
Concentration factors are relatively low under these experimental conditions. Following 5 days' exposure, males had a mean concentration factor of 11.3 x. The comparable concentration factor for females is 59.3 x. The mean concentration factors for both males and females were lower following 10 days' exposure, suggesting that a critical maximum level of toxicant had been reached. Organisms do not accumulate and retain the substance to the same extent as they do a more stable compound.

Table 10. Whole-body HCBD residue concentrations in male and female crayfish (Procambarus clarki)during uptake and depuration. Concentrations in µg/Kg (ppb).

		Days Exposure			Days Depuration		
	HCBD (ug/Kg) in tissue	1	5	10	1	5	10
TANK A 2.2 ppb HCBD	Males	2.5	59.8	13.8	17.7	1.2	34.1
	Females	12.3	87.6	99.8	20.7	9	10.8
TANK B 3.7 ppb HCBD	HCBD (ug/Kg) in tissue						
	Males	3.4	7.1	41.1	49.4	1.0	69.6*
	Females	3.8	264.4	14.3	19.4	141.2	15.9
TANK C H_2O Control	HCBD (ug/Kg) in tissue						
	Males	15.2	1.8	20.9	23.8	19.7	1.0
	Females	42.1	3.0	22.2	75.8	8.9	1.3
TANK D Acetone Control	HCBD (ug/Kg) in tissue						
	Males	17.6	2.8	37.1	22.8	14.2	.9
	Females	4.0	12.6	19.4	23.2	51.4	.9

* single specimen

Figure 14. Uptake and depuration of HCBD in crayfish (Procambarus clarki) exposed to a mean concentration of 3 ppb (µg/ℓ). Average values* from Table 10.



* four animals per data point

2. Mollies

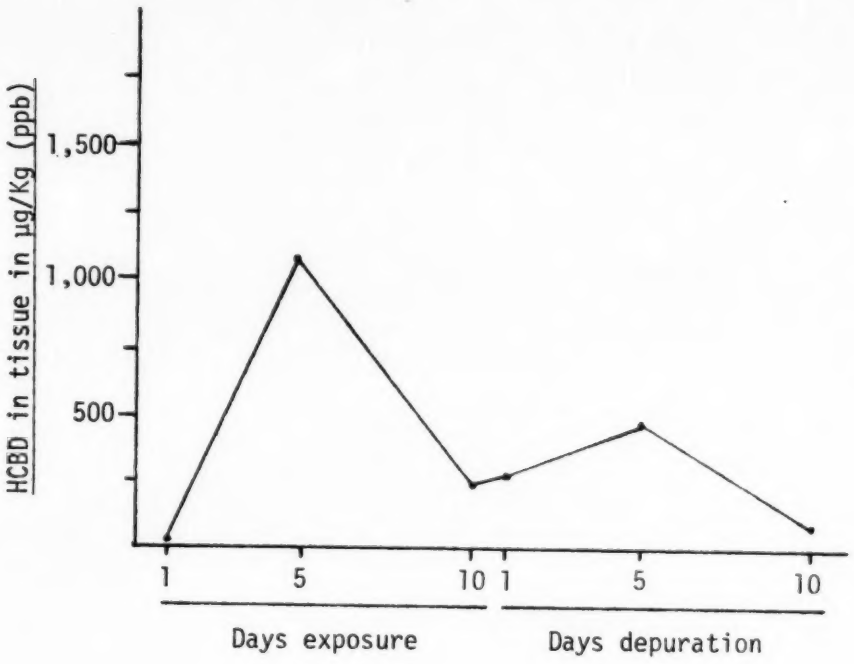
Mollies were subjected to nominal concentrations of 10 ppb HCBD and control conditions in the diluter, to provide uptake and depuration information. As shown in Table 11, uptake does not proceed steadily, but rather it reaches a maximum in less than one week. Further exposure does not appear to increase the level of HCBD in test animals, and concentration factors remain relatively low. A low level of HCBD contamination was found in control tanks, probably resulting from diffusion of the volatile substance through the air. The same low concentration of HCBD is also seen in controls listed in Table 11.

Figure 15 shows the erratic pattern of uptake and depuration of HCBD in mollies. Each data point represents two separate GC analyses of an aggregate of four males and two females.

Table 11. Whole-body HCBD residue concentrations in sailfin mollies (Poecilia latipinna) during uptake and depuration. Concentrations in µg/Kg (ppb).

		Days exposure			Days depuration		
		1	5	10	1	5	10
Tank A 10ppb (nominal) 10.54 ppb (GC)	HCBD (ug/Kg)	783	2,066	239	140	61	94
	Sample wt (g)	5.713	7.639	6.992	7.242	4.948	6.743
	Concentration factors	74.3x	196x	22.7x			
Tank B 10ppb (nominal) 12.03 (GC)	HCBD (ug/Kg)	20	294	273	431	925	118
	Sample wt (g)	6.510	7.402	7.785	5.944	6.129	5.326
	Concentration factors	1.7x	24.4x	22.7x			
Tank C Water Control(.97ppb)	HCBD (ug/Kg)	25	52	246	4	14	133
	Sample wt (g)	7.791	5.832	6.400	6.903	5.945	4.658
	Concentration factors	25.8x	53.6x	253.6x			
Tank D Acetone Control(1.56ppb)	HCBD (ug/Kg)	26	19	117	123	209	124
	Sample wt (g)	6.887	8.175	7.039	7.141	4.598	6.737
	Concentration factors	16.7x	12.2x	75x			

Figure 15. Uptake and depuration of HCBD in sailfin mollies (Poecilia latipinna) exposed to a mean concentration of 11.3 ppb (µg/ℓ). Average values from Table 11.



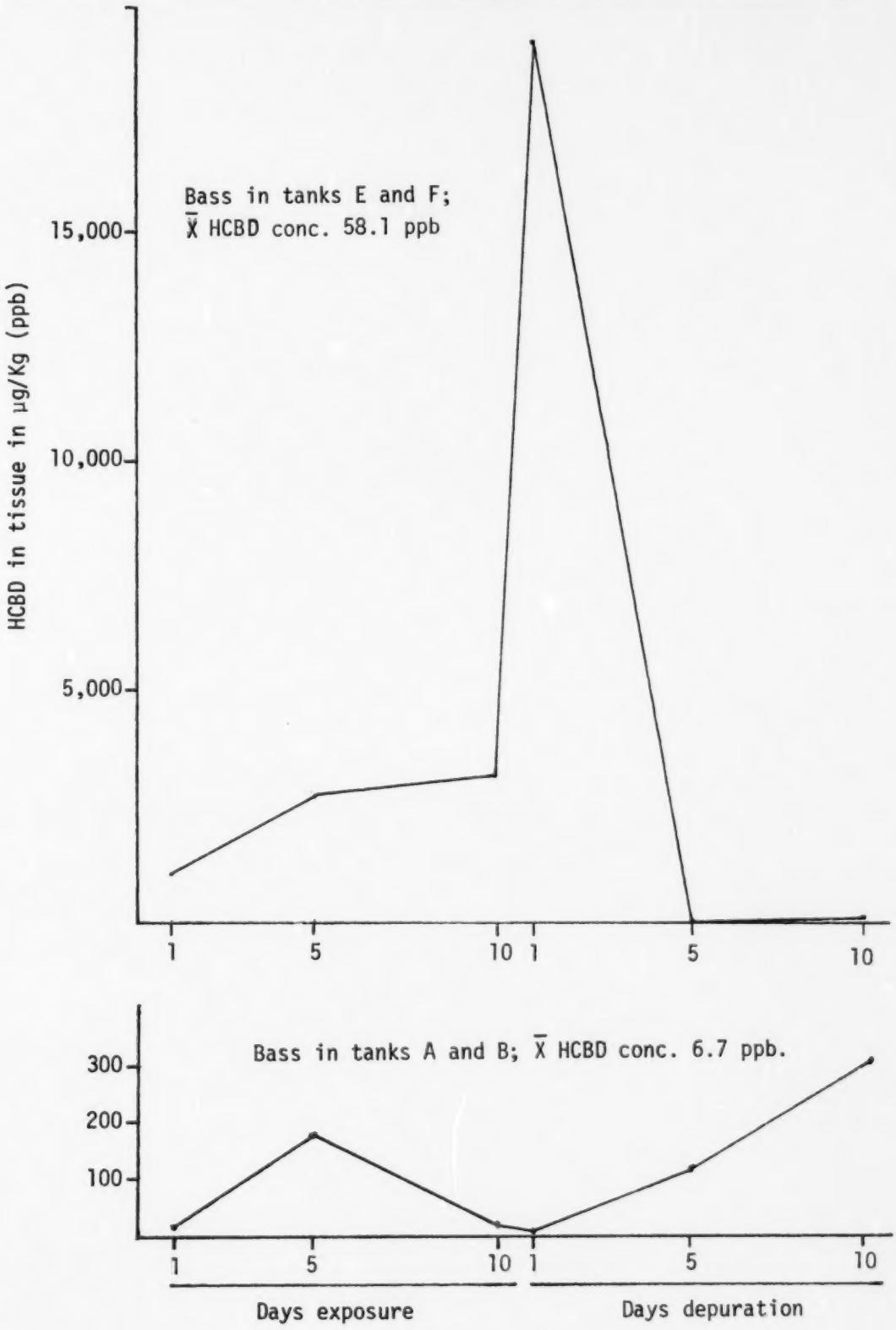
3. Bass

Table 12 gives concentrations of HCBD in bass tissue during both uptake and depuration. As has been observed with other organisms, uptake rate is not consistent in whole body samples. Concentration factors remain relatively low, reaching a maximum of 112X in this experiment, and do not correlate with size (weight) of the test organism. The levels accumulated, however, do reflect the concentration of HCBD in the environment, within limits. Figure 16 shows this and illustrates the irregularity with which specimens responded to the compound. Increases in levels during depuration are not readily explained.

Table 12 . Whole-body HCBD residue concentrations in largemouth bass (Micropterus salmoides) during uptake and depuration.

		Days exposure			Days depuration		
		1	5	10	1	5	10
TANK A 5.65ppb	HCBD (ug/Kg) in tissue	23.7	277.9	25.0	6.9	6.7	546.2
	Mean wt (g)	1.73	2.36	3.27	3.65	3.74	5.70
	Concentration factors	4.2x	49.6x	4.4x			
TANK B 7.8ppb	HCBD (ug/Kg) in tissue	10.7	69.1	32.6	9.0	223.6	109.6
	Mean wt (g)	2.79	2.99	3.22	5.13	8.16	6.53
	Concentration factors	1.4x	8.9x	4.2x			
TANK C 4.86ppb	HCBD (ug/Kg) in tissue	10.8	19.2	10.6	7.7	167.3	2.4
	Mean wt (g)	3.29	3.02	4.06	6.21	6.84	12.94
	Concentration factors	3.2x	4.0x	2.2x			
TANK D 4.36ppb	HCBD (ug/Kg) in tissue	13.9	8.1	83.2	12.4	7.2	1.3
	Mean wt (g)	3.45	5.04	4.71	3.86	5.28	10.32
	Concentration factors	3.2x	1.9x	19.1x			
TANK E 59.1ppb	HCBD(ug/Kg) in tissue	91.9	5,041.3	145.9	12,422.3	52.5	13.8
	Mean wt (g)	3.74	4.02	4.26	5.77	6.06	7.93
	Concentration factors	1.6x	85.3x	2.5x			
TANK F 57.12ppb	HCBD (ug/Kg) in tissue	1,852.6	86.7	6,446.4	25,372.5	4.5	291.1
	Mean wt (g)	2.66	5.21	6.89	4.42	5.61	7.27
	Concentration factors	32.4x	1.5x	112.9x			

Figure 16 . Uptake and depuration of HCBD in bass (Micropterus salmoides). Average values from Table 12.



4. Algae

In a flow-through experiment with green filamentous algae (Oedogonium cardiacum), higher concentration factors were observed than in laboratory tests with other organisms. Results of an accumulation experiment are given in Table 13.

Table 13. Concentration of HCBD by a green alga, Oedogonium cardiacum exposed to a flowing solution of 16.9 ppb HCBD in water.

Days exposure	HCBD in µg/Kg (ppb) (wet weight)	Concentration factor
1	965.8	57.1x
3	2,547.2	150.7x
7	2,700.6	159.8x

5. Bottom Sediment

In a brief laboratory experiment, soil was found to accumulate HCBD more readily than did experimental animals. Following one day of exposure to a regular flow of 3.6 ppb HCBD, a sediment sample contained a concentration of 725 µg/Kg HCBD (725 ppb), a concentration factor of 201.4x. Four days after initiation of the test, soil contained 938 ppb HCBD, a concentration factor of 260.5x. Depuration began on the fourth day. After four days of depuration 632 ppb of HCBD were measured by GC in the sediment.

6. Effect of Food Chain

Experiments with HCB show a significant difference between amount of the compound taken in by bass from water sources in comparison with HCB included in their diet alone. A similar test was made with HCBD to determine if that compound showed a similar pattern in bass. Five bass were placed in each of two test tanks. Tank 1 received an average concentration of 3.0 ppb HCBD. Tank 2 was a water control. Bass being tested were fed ad libitum HCBD-contaminated mollies containing approximately .02 µg/g (20 ppb) HCBD for seven of the nine days of the experiment. All bass were fasted the last two days to permit clearance of all solid food material from the gut. Results of this experiment are given in Table 14.

Table 14. HCBD concentrations in largemouth bass (Micropterus salmoides) feeding on contaminated sailfin mollies (Poecilia latipinna).

Bass held in 3 ppb HCBD			Bass held in HCBD-free water		
Bass spec. #	wt in g	HCBD in µg/Kg (ppb)	Bass spec. #	wt in g	HCBD in µg/Kg (ppb)
1	24.5	802.4	1	15.2	4.1
2	26.0	5.0	2	18.2	3.8
3	26.3	2,227.4	3	20.3	50.0
4	34.0	3.3	4	36.9	11.8
5	40.6	1.2	5	42.4	2.7

These data draw attention to the great variability in the uptake of HCBD by individual organisms. No useful trends are apparent in a sample of this size, except that, on the average, bass in contaminated tanks accumulated more HCBD than their counterparts which were fed contaminated mollies in control water.

7. Crayfish Uptake of HCBD in Field Environment

To provide broad information concerning uptake of HCBD (and HCB) in a field situation, a group of crayfish was caged and placed in a waste disposal pond at the site of the Vulcan Materials Company at a time when HCBD concentrations were 4.3 to 4.6 ppb in water and 381.7µg/Kg (381.7 ppb) in mud. At the end of 17 days' exposure, the mean concentration of HCBD in eight mature crayfish was 438 ppb (range 33.7 - 1,290.3 ppb). Distribution of results is given in Table 15.

Table 15. HCBD content of crayfish exposed to 4.3 ppb HCBD in a field locality.

Number of Specimens	Mean HCBD concentration in µg/Kg (ppb)	Concentration factor
4	93	22X
1	357	83X
1	443	103X
1	1,042	242X
1	1,290	300X

Three other crayfish exposed to similar conditions for five days in the same field site contained 9, 33, and 240 ppb HCBD which represent concentration factors of 2.0X, 7.2X, and 55.8X.

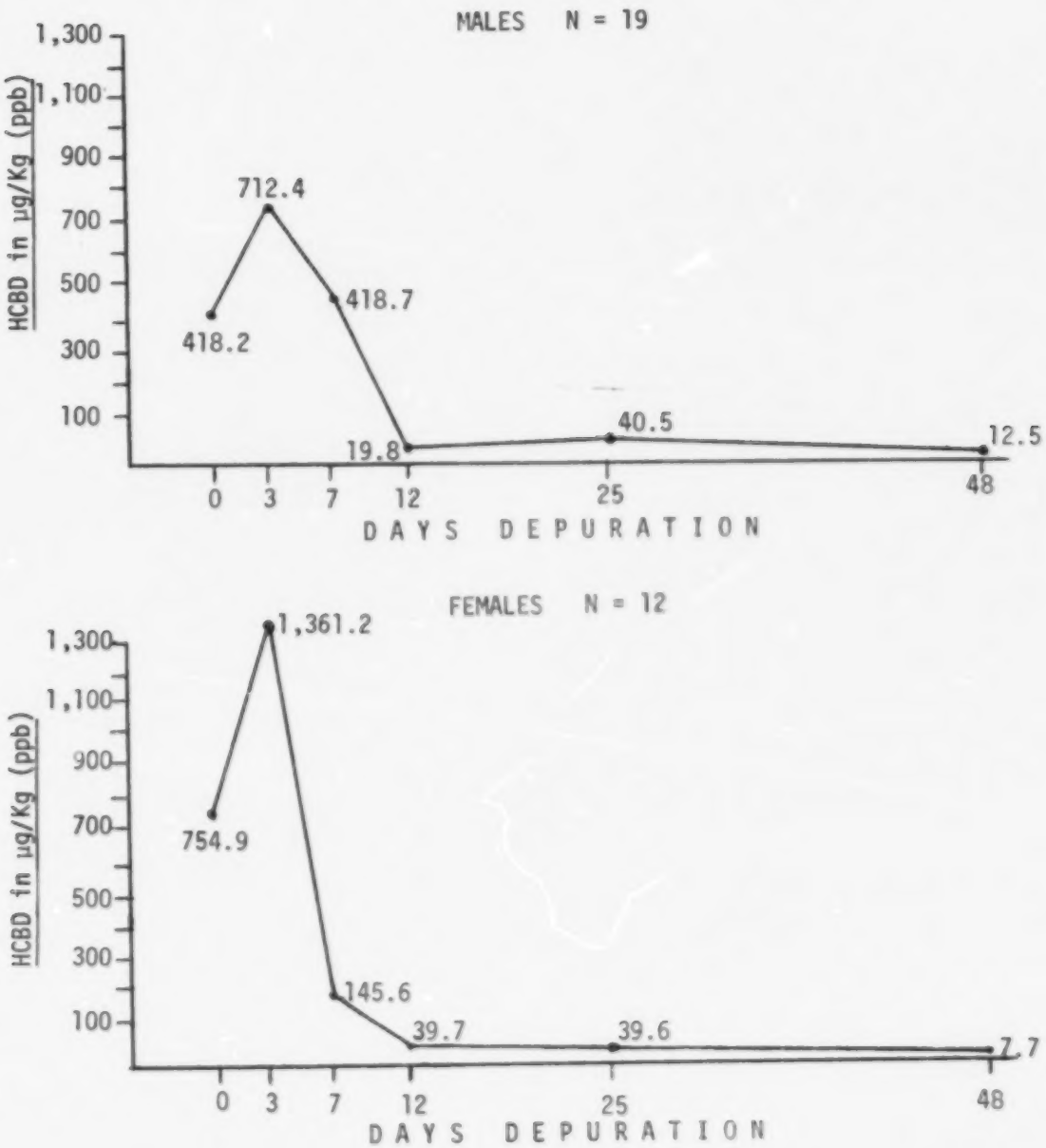
Six crayfish were exposed for different periods and depurated either not at all or for two months. Data resulting from this part of the study are presented in Table 16. HCBD concentration in water was 4.6 ppb and in mud was 381.7 ppb. The variation both in uptake and depuration rates is readily seen in this table.

Table 16 . Short and long-term exposure of crayfish to a contaminated field site (4.6 ppb HCBD).

Days exposure	Days depuration	Weight (in g) of specimen	HCBD Concentration in µg/Kg (ppb)
4	63	19.32	8
4	63	13.93	132
15	63	17.39	186
51	0	12.30	501
51	0	13.55	320
51	0	24.61	394

Thirty-one crayfish were placed at the field site where they were exposed to 4.7 ppb HCBD for 10 days. A sample was sacrificed on removal from the field. Remaining animals were depurated in environmental chambers in the laboratory, and samples were sacrificed for GC analysis on days 3, 7, 12, 25 and 48 of depuration. The results, given in Figure 17, show a higher level of uptake by females and a rapid loss of the substance by both sexes. The samples having highest levels of HCBD, sacrificed on day three of depuration yielded concentration factors of 151X (712 ppb) in males and 289X (1,360 ppb) in females.

Figure 17 . Levels of HCBD in crayfish during depuration following 10 days exposure to a contaminated environment (4.7 ppb HCBD).



E. Respirometry

1. Crayfish

Experiments were designed to measure possible effects of HCB upon respiration in crayfish. The average oxygen consumption rates ($\mu\ell$ O_2/mg wt/hr) for HCBD-exposed and for control animals are shown in Table 17. Each value is based upon an average of 20 animals whose oxygen consumption rates were measured for a total time period of two hours. In Figure 18 the percentage deviations of the experimental groups from control groups are plotted against time (in days). Time "zero" represents the initial exposure period. By establishing the control values as a base line, any trends in the experimental values should become evident through this method of plotting the results.

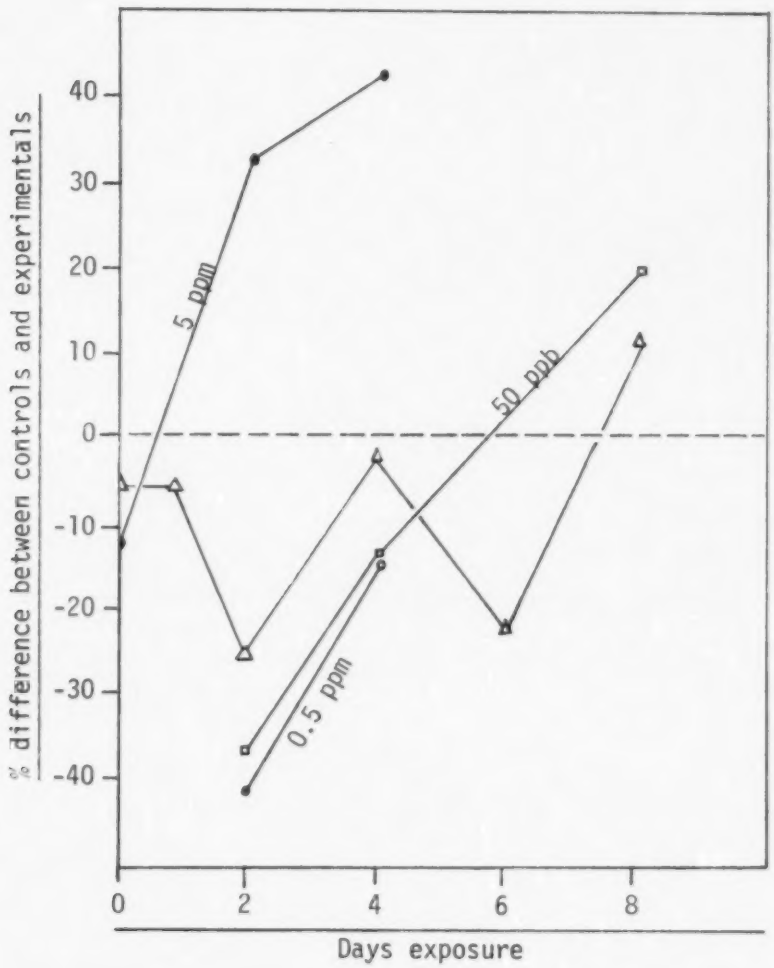
Exposure of juvenile crayfish to 5 ppb HCBD shows no significant effect upon oxygen consumption rate during the first two hours of exposure. Chronic exposures to 5 and 0.5 ppm and 50 ppb for periods as long as eight days produced complex patterns which are difficult to interpret until more extensive studies are completed. A preliminary pattern which is emerging (Fig. 18) indicates that respiration rate may be initially depressed and then accelerated after several days of exposure.

An HCBD concentration of 5 ppm was selected for the first experiment because it was known to be an acute lethal dose in crayfish. At 5 ppm half of the juveniles in our experiment died within 72 hours. The HCBD concentration was reduced to 0.5 ppm, and fifty percent of the animals died within 120 hours. At 50 ppb the juvenile crayfish survived indefinitely.

Table 17. Effect of time of exposure to HCBD on respiration rate of juvenile crayfish ($\mu\ell$ O_2/mg/hr).

Experiment #	Group	Duration of exposure (in days)					
		0	1	2	4	6	8
1	Control	0.3866		0.3439	0.3116		
	Exp. (5 ppm)	0.3434		0.4550	0.4419		
2	Control			0.4613	0.4532		
	Exp. (0.5 ppm)			0.2488	0.3852		
3	Control			0.5725	0.5614		0.3265
	Exp. (50 ppb)			0.3680	0.4856		0.3872
4	Control	0.2746	0.1835	0.3817	0.3171	0.2562	0.1931
	Exp. (50 ppb)	0.2608	0.1749	0.2871	0.3111	0.1793	0.2143

Figure 18. The effect of time of exposure to HCBD on respiration rate of juvenile crayfish (Procambarus clarki).



2. Mollies

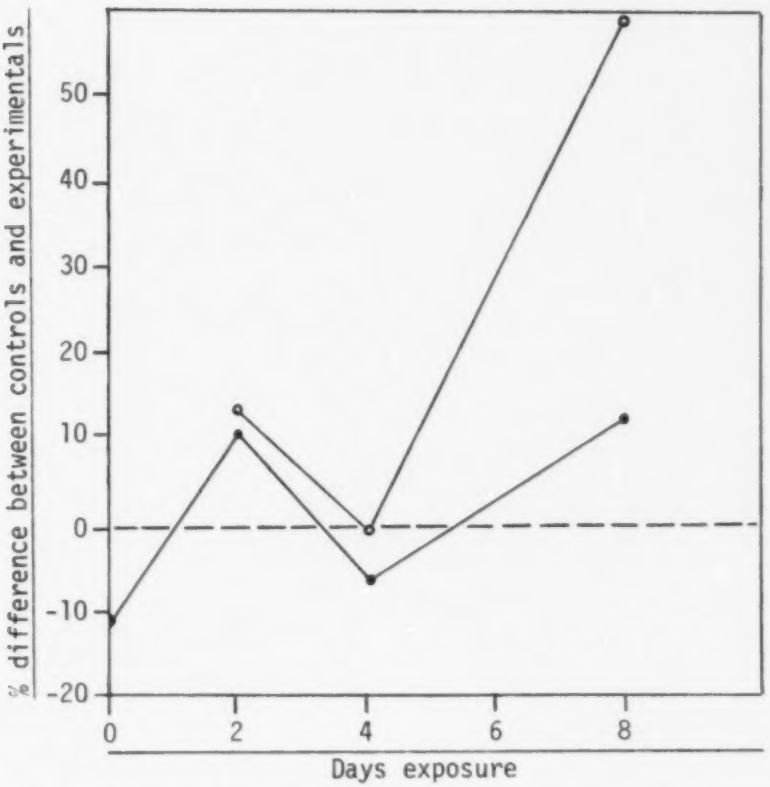
Experiments were carried out to determine the possible effect of HCBD upon oxygen consumption in mollies (Poecilia latipinna). The average oxygen consumption rates ($\mu\ell$ O_2/mg wt/hr) for HCBD-exposed and for control animals are shown in Table 18. Each value is based upon an average of 4 animals whose oxygen consumption rates were measured for a total time period of one hour. In Figure 19 the percentage deviations of the experimental groups from control groups are plotted against time (in days). Time "zero" represents the initial exposure period. By establishing the control values as a base line, any trends in the experimental values should become evident through this method of plotting the results.

Juvenile mollies were exposed to 0.5 ppm HCBD and oxygen consumption determined following exposure periods as long as eight days. Figure 19 fails to show a consistent pattern of response in the experimentals. Because of the variable responses observed, much more data is needed before a pattern of response can be described.

Table 18. Effect of time of exposure to HCBD (0.5 ppm) on respiration rate of juvenile mollies ($\mu\ell$ O_2/mg/hr).

Experiment #	Group	Duration of exposure (in days)			
		0	2	4	8
1	Control		0.4750	0.4685	0.3778
	Exp.		0.5240	0.4404	0.3692
2	Control	0.3022	0.4277	0.2932	0.3905
	Exp.	0.2707	0.4856	0.2960	0.6212

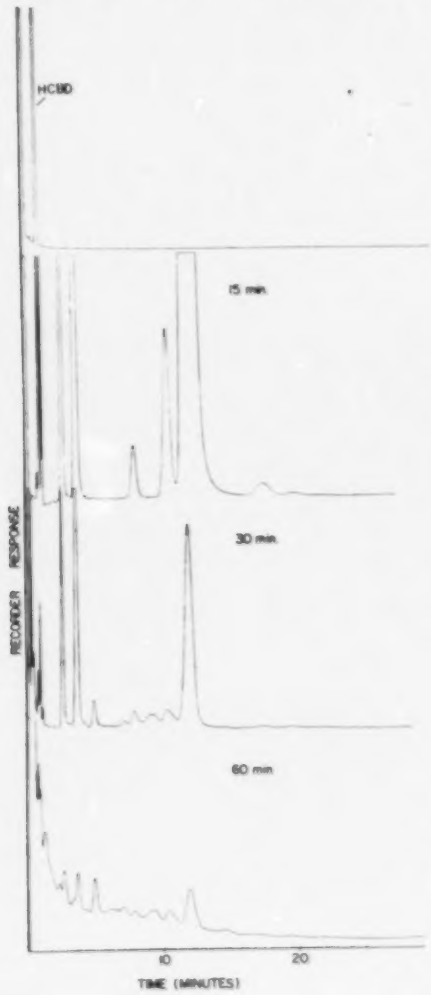
Figure 19. The effect of time of exposure to HCBD (0.5 ppm) on respiration rate of juvenile mollies (Poecilia latipinna).



F. Photochemistry

HCBD in benzene shows a dramatic change upon irradiation. After only 15 minutes of irradiating at a wavelength of 2735 Å , numerous products having a molecular weight higher than HCBD are evident in large quantities relative to the initial amount of HCBD. The number of these products decreases with the time of exposure. GC traces of resultant products taken at intervals are shown in Figure 20.

Figure 20. Gas chromatographic separation of photoproducts resulting from ultraviolet irradiation of HCBD.



VI. Discussion of Results

Analytical support for the studies contained in this report included GC analyses of more than 1,300 separate water, soil and organism samples. Laboratory specimens accounted for more than 1,000 of these prepared extracts, while the remaining analyses included samples collected during more than 40 field trips.

During the field phase which encompassed a transect along the Mississippi River from Baton Rouge to Port Sulphur, Louisiana, HCBD residues were found in several soil and water samples. The level in water did not reach 2 µg/ℓ (ppb), but concentrations of HCBD in mud or soil samples exceeded 200 µg/Kg (ppb) between Ashland Plantation and Romeville and at New Orleans. All these sites were along the east bank of the Mississippi River. Significant levels of HCBD were also found at Addis, on the west bank and at Myrtle Grove, below New Orleans, also on the west bank.

Substantially greater loads of HCBD were found in mud samples when compared with HCBD concentrations in most organisms (mosquitofish, Gambusia affinis, and crayfish, Procambarus sp.) taken from field sites along the Mississippi River. At Romeville, for instance, river water had a level of 1.4 ppb, soil had 793.4 ppb and fish had 197.4 ppb HCBD. Concentrations of HCBD in river samples tended to be more variable than those from ditch sites.

Variable patterns of generally low-level contamination of HCBD were seen as a result of seasonal collections from localities at the area of higher concentration in Geismar, Louisiana. Most water samples contained less than 1 ppb HCBD and none had more than 5 ppb. Levels in mud were noticeably higher, reaching a maximum of 2,370 ppb. Organism samples contained less than 1000 ppb HCBD with few exceptions. Gambusia, the mosquitofish, had an average of 16,000 ppb HCBD. These specimens came from a pond of highest contamination, where concentrations in water ranged from 4 to 5 ppb. Observations of HCBD levels in the Mississippi River survey and the more limited Geismar area corroborate one another as much as might be expected with the variability seen in HCBD. There is a general association of HCBD with the Mississippi River. The principal source of contamination is very likely to be petrochemical industries that border the river.

Studies of HCBD acute toxicity in test animals have emphasized uptake by oral administration, injection, and topical application. Gul'ko et al. (1965) reported LD_{50}'s of 41 mg/Kg (ppm) in mice, taken orally. Topical applications of 500 mg of HCBD to the shaven skin of guinea pigs caused intoxication in some, as well as some deaths (Murzakaev, 1966).

Murzakaev (1963a) also performed injection experiments with mammals. He reported LD_{50}'s resulting from single intraventricular injections of 87,90 and 350 mg/Kg (ppm) in mice, guinea pigs and rats, respectively. Poteryaeva (1966) reported mortalities in offspring of white rats born up to three months after the mothers had been injected subcutaneously with 20 mg HCBD/Kg body wt.

Injection experiments in the present study used crayfish (Procambarus clarki) and Gulf killifish (Fundulus grandis). Injection of 3.4 mg HCBD per gram body weight (ppt), in peanut oil as a carrier, was a 24-hour LD_{50} for crayfish. A

five-day LD_{50} was 336 µg/g. In killifish, intraperitoneal injection of 3.4 mg HCBD/g (ppt) was a four-hour LD_{50}. A 1.7 ppt injection was a 20-hour LD_{50}. An LD_{50} of 18 days was 800 µg/g (ppm), but no fish deaths were noted during a three-week period at a dosage of 200 ppm. The greater toxicity of HCBD to crayfish may be in part due to the site of injection. Crayfish were injected directly into the hemocoel, permitting more rapid circulation throughout the body. Killifish were injected into the peritoneal cavity, and some of the oil was observed to have been lost from the fish when they were released into tanks. Therefore, killifish might not have retained the full dosage administered and the distribution in the body may have been slow.

Very little work has been published on toxicity of HCBD to aquatic animals. Hiatt et al. (1953) noted a behavioral response in schooling fish to applications of 20 ppm (mg/ℓ). HCBD had been dispersed but was not necessarily dissolved in water. Stroganov and Kolosova (1968) exposed cyprinid fish, Leucaspius delineatus to toxic levels of HCBD in water and reported total mortality even when, after a brief exposure to the toxicant, the fish were transferred to clean water and showed some recovery. They stated that concentrations below 15.6 ppm had no toxic effects, even after 11 days of exposure. Experiments carried out during the present study found HCBD to be acutely toxic at lower concentrations than previously reported. A 54-hour LC_{50} for crayfish held in the diluter was 2.9 ppm.

Bass were not overtly affected by 10 days' exposure to 3.43, 31.95, or 59.10 ppb (µg/ℓ) HCBD in the diluter. At a level of 900 ppb (.9 ppm) bass were distressed in 24 hours but survived more than four days under these conditions, although they were inverted and immobilized. Some variability was noted in tests with bass. Concentrations of 2.5 ppm and 4.3 ppm were 24- and 48-hour LC_{50}'s, respectively. Larger numbers of mollies were available for acute experiments. LC_{50}'s for 26 and 30.5 hours were 4.2 ppm and 4.5 ppm HCBD, respectively. Little difference was apparent in the response of the two species to HCBD. It should be noted, however, that molly experiments took place in 2.5 ppt salinity, while 1.0 ppt salinity water was used for bass. Furthermore, bass were substantially larger than mollies.

More subtle effects of chronic doses of a substance are expected to be seen at the tissue and cellular level. Reports of damage at the histological level to aquatic animals exposed to chronic doses of HCBD have not been published. However, microscopic observations of morphological changes following HCBD exposure are reported for the rat in liver (Gul'ko et al., 1965 and Murzakaev, 1967) and in kidney tissues (Dmitrienko et al., 1972; Gage, 1970; and Murzakaev, 1967). In histological sections of crayfish tissue prepared during the course of the present study, abnormalities in site and degree of hepatopancreas epithelial exfoliation were noted. This change and the increased mitotic rate and indications of disorganization in growth in the germinative tips suggest possible chronic effects of an exposure to 2.97 ppb (µg/ℓ) HCBD for 10 days. Analyses of crayfish tissue extracts on GC showed the brain as having the highest concentration of HCBD, (35 µg/g) with the hepatopancreas having 200 times less HCBD residue, (.173 µg/g). The green gland has a concentration an order of magnitude higher than the hepatopancreas; 2.188 µg/g (ppm).

Histological slides of tissue from bass exposed to 32 ppb for 10 days showed damage tc liver parenchyma, gall bladder, and kidney. In GC analyses of extracts from these tissues, highest HCBD residue concentration was found in gut, followed by liver and kidney.

Although to date there are relatively few animals in each category that have been submitted to a thorough histological scanning, the findings are so consistent that continued examination of the accumulated tissue blocks is warranted. Furthermore, repetition of diluter exposure experiments and subsequent depuration with histological sampling to determine the extent of tissue recovery would lend further significance to the findings.

In addition to histological preparations of bass tissue, a considerable volume of material has been prepared from a second fish species, the sailfin molly (Poecilia latipinna). Mollies had been used in several experiments during the present study, and substantial information on their response to the test compounds has been included in this report. Observations of gross pathology of the liver and gall bladder have been noted and warrant microscopic examination of the accumulated material. It seems particularly appropriate to continue to explore the liver and gall bladder response to HCBD noted in the bass and molly in light of the findings of cholecystitis in vineyard workers having contact with HCBD as a fumigant (Krasnyuk et al., 1969).

The extent to which a substance is accumulated by an organism during prolonged exposure to it may have an effect upon health and survival. Concentration and period of exposure may influence both the maximum level attained and the time required for depuration. Laboratory experiments included in this report exposed organisms to HCBD in both static and flow-through systems. In general, concentrations in whole organisms increased with time of exposure and decreased with time of depuration. Rates of accumulation and depuration varied widely between organisms.

In laboratory experiments, crayfish accumulated relatively small amounts of HCBD. Concentration factors in females were higher than in males, but did not exceed 60X during 10-day exposures to a mean of 2.97 ppb (µg/ℓ). In mollies exposed to a mean of 11.29 ppb, concentration factors reached 110X during a 10-day test period. Bass held in tanks with 6.73 ppb HCBD had a maximum concentration factor of 29X during a 10-day exposure. The lower concentration factors of bass in comparison with mosquitofish may in part be explained by Murphy's (1971) observation, that smaller organisms in aquatic systems accumulate proportionately greater amounts of a toxin than larger ones do. Bass were, on the average, 2 to 4 times larger than mollies at the time of their respective experiments.

Crayfish in laboratory experiments accumulated proportionately less HCBD than mollies did. In the field, crayfish accumulated proportionately less than mosquitofish, a member of the same fish family, Poeciliidae, as mollies. It appears that either the fishes are more efficient at taking in and concentrating the substance or the crayfish have more effective means of altering the structure of accumulated HCBD. A combination of these factors may be at work. Mosquitofish at the Geismar site accumulated higher levels and had higher concentration factors than mollies tested in the laboratory. This may be a result of extended periods of exposure time.

Algae samples in the laboratory accumulated HCBD at higher levels than animals did. After 7 days of exposure to 16.9 µg/ℓ (ppb), the level attained was 2,700 µg/Kg (ppb); a concentration factor of 160x. Sediment was subjected to 3.6 µg/ℓ (ppb) HCBD. The highest level attained was 938 µg/Kg after four days; a concentration factor of 261x. Depuration was gradual, since 632 ppb of HCBD remained in a sample taken after four days. The highest level of HCBD found in soil at Geismar was 2,370 µg/Kg (ppb).

A food chain experiment was designed to compare the effect of HCBD uptake by ingestion with the combination of uptake by ingestion and through a contaminated environment. A considerable variability was observed among individual organisms and results were inconclusive. Only when considered on the average did bass in HCBD-contaminated tanks accumulate more HCBD than those which were fed contaminated mollies in clean water.

Experiments in which HCBD-free crayfish were caged and placed in a field site with known HCB and HCBD contamination provided a more natural situation for observing uptake rates. Mean concentration factors and individual variability were similar to those for crayfish tested in the laboratory. Crayfish exposed to 4.6 ppb HCBD in pond water for 17 days accumulated a wide range of residue concentrations. The mean level was 438 µg/Kg (ppb), but the range was 33 to 1,290 ppb. The concentration factors for these were from 7X to 300X. Females accumulated more of the compound than did males, and HCBD levels in both sexes decreased at a rapid rate between days 3 and 12 of depuration. In animals held in the field site for 10 days, approximately 95% of the HCBD measured at the onset of depuration was lost within 12 days.

Laboratory experiments revealed physiological effects upon test animals subjected to HCBD. Stress by HCBD in water was indicated as a result of analysis of corticosteroid level in blood. Plasma cortisol levels in bass exposed to 3.43 and 31.95 ppb (µg/ℓ) HCBD for 10 days were significantly higher than corresponding levels in controls (5% level).

As a further test for parameters indicating stress due to a chronic exposure, several experiments were carried out in which juvenile mollies and crayfish were subjected to HCBD in water and their oxygen consumption rate monitored in a Gilson respirometer. HCBD seemed to have no significant effect upon oxygen consumption rate of crayfish during a two-hour exposure to 5 ppb HCBD. Complex patterns of response were seen in crayfish exposed to 50 ppb and .5 and 5 ppm HCBD for extended periods of time as long as 8 days. Tentative results show an initial depression in rate followed by an accelerated rate after several days. Experimental mollies in these studies had extremely variable response patterns. A great deal more data is essential before any definitive statements can be made on this subject.

Oxygen consumption studies utilized the Gilson Differential Respirometer instead of a flow-through respirometer which we designed, because of several advantages which became evident during our experiments. During the season of the year when the flow-through system was completed we had available only small juvenile animals which could not be used in a flow-through respirometer. The Gilson Respirometer was more sensitive. Significant differences between control and experimental animals did not appear within 24 hours. The flow-through system was designed to measure oxygen consumption during periods up to 12 hours. Bass did not survive in the confined chambers of the flow-through system. These circumstances, among others, made the Gilson unit a more useful instrument.

The results of respiration measurements both in mollies and crayfish did not produce repeatable patterns from one experiment to another. It must be concluded that, in the case of HCBD, oxygen consumption data from whole animals was not particularly useful as an indication of chronic stress.

The data, presented as percentage variation of the experimental from control rates, in Figures 18 and 19 often indicated high percentage differences. HCBD

is a hydrophobic substance. Its distribution may be through passive partitioning through lipid phases in the organism. Any effects on respiration may be a result of the accumulation of HCBD in membranes. Many enzymes depend upon the integrity of the membrane elements in the immediate vicinity of the enzyme (Coleman, 1973). Steric distortion of membranes by hydrophobic substances which partition into membranes may be an important and chronically cumulative problem in toxicology. The effects on a central metabolic process, such as oxidative phosphorylation, may be complex. For example, if steric alteration of membranes in the vicinity of the electron transport system in mitochondria occurs, the transport system may be inhibited. Oxygen utilization would be decreased. If, on the other hand, oxidative phosphorylation were uncoupled from electron transport by the steric alteration, oxygen utilization would increase. The absence of rate changes upon initial exposure may reflect the time required for transfer into the organism. Variations in exposure time and concentrations of hydrophobic substances such as HCB for longer exposure periods may result in highly complex effects when the whole organism is studied. Respiration studies utilizing mitochondria and specific experiments utilizing membrane-bound enzymes may produce much more useful results. Such experiments are particularly recommended in view of the tissue alterations which we have reported in the present study.

An intensive investigation of cellular and subcellular damage in critical organs utilizing electron microscopy in combination with subcellular physiological and enzymatic studies would be an important direction in the toxicology of hydrophobic substances. Establishment of detailed syndromes of toxicological effects which are legally defensible are much more likely at the subcellular level, than at the organismic level. It is expected that early warnings of toxic effects would first be observed at the levels of enzymatic activity and subcellular structure. We recommend this direction as potentially important.

Pertinent information from acute toxicity experiments is summarized in Table 19. Data from injection and diluter exposures are included. Table 20 presents an overview of the critical results from this project. The table includes only those experimental results which clearly showed some alteration in experimental animals. From each of those experiments only the minimum concentration resulting in the effect is included in the table. Maximum observed concentration in the environment at Geismar and along the Mississippi River are included for comparison purposes.

Table 19. Summary of acute toxicity experiments with HCBD.

	Organism	Mode of exposure	Concentration	Total # of organisms exposed	Duration of exposure	Duration of observation	% Mortality
Injection Experiments	Procambarus clarki (Crayfish)	single injection	34 µg/g body wt	10	single injection	6 days	30
	"	"	336 "	10	"	6 "	100
	"	"	3,360 "	10	"	2 "	100
	Fundulus grandis (Gulf Killifish)	"	200 µg/g body wt	10	"	21 "	-
	"	"	800 "	10	"	18 "	60
	"	"	1,700 "	10	"	22.5 hr	100
	"	"	3,400 "	10	"	4 hr	100
Diluter Experiments	Procambarus clarki (Crayfish)	aqueous solution in diluter	2.7 ppm	9	12 days	12 days	100
	"	"	2.9 "	9	12 "	12 "	100
	Poecilia latipinna (Sailfin molly)	"	1.2 "	11	6 "	"	100
	"	"	1.4 "	16	6 "	"	100
	"	"	1.7 "	14	6 "	9 days	100
	"	"	1.9 "	11	5 "	5 "	100
	"	"	4.2 "	20	5.5 "	5.5 "	100
	"	"	4.5 "	20	5 "	5 "	100
	Micropterus salmoides (Largemouth black bass)	"	.9 "	6	4 "	8 "	-
	"	"	2.2 "	6	4 "	4 "	100
	"	"	2.5 "	6	1 "	1 "	100
	"	"	4.3 "	6	1.5 "	1.5 "	100

Table 20. Minimum concentrations of HCBD tested that resulted in an observed response in organisms.

Method	Lowest tested level (in ppb) at which changes were observed	Observations	Section containing discussion
a) Direct injection in oil medium	34,000. µg/Kg body wt	30% mortality to crayfish in 6 days following injection	V B1
	800,000. µg/Kg body wt	18-day LD_{50} for injected Gulf killifish	V B2
b) Diluter	2,700.	57-hour LC_{50} for crayfish	V B3
	900.	Distress within 24 hours for bass	V B4
	1,200.	138-hour LC_{50} for bass	V B4
	4,200.	26.5-hour LC_{50} for mollies	V B4
c) Histology	3.	Heightened rate of mitosis in germinative tips of hepatopancreas; Distal epithelial exfoliation in crayfish.	V C1
	33.	Accented lobulation of liver parenchyma Leukocytic infiltration of kidney in bass	V C2
d) Cortisol level in blood plasma	3.	Cortisol level in blood elevated (Significant at 5% level) in bass	V C6

Maximum HCBD concentrations found in environmental survey

	Water	Mud *
Area of Higher Concentration	4.7 ppb (µg/ℓ)	1,080. (2,370.) ppb (µg/Kg)
Overview	1.9 ppb (µg/ℓ) (Baton Rouge)	790. (1,088.) ppb (µg/Kg) (Romeville)

*: Figures corrected for dry weight in parentheses.

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